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HYPOTHYROIDISM CAUSES CELLULAR DAMAGE IN RETINAL TISSUE AND BICEPS IN RATS

Manalee Guha2, Preeta Sen2, Sudakshina Ghosh2, Soumeet Ghosh2, Anisha Chaudhuri2, Pritha Gupta2, Sontirtha Das2, Sweta Mukherjee2, Reni Ghosh2, Rini Roychowdhury2, Srikanta Guria1, and Madhusudan Das2*#

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ABSTRACT

Thyroid hormones regulate a number of physiological functions in the body (Fujita, 1988). We hypothesized thyroid hormone may have an important role in regulating the function and cytomorphology of retina of eye and bicep muscle in rat. We treated experimental group of rat with methimazole intraperitoneally to induce hypothyroid like conditions. Methimazole induced hypothyroid rat exhibited drastic changes of retinal tissue. Hypothyroid rat exhibited the changes in the architecture of rat biceps. Therefore, hypothyroidism may be an important cause for visual problem as well as thyroid myopathy.

Keywords: hypothyroid, methimazole, thyroxine, biceps, retina

INTRODUCTION

Thyroid hormones control a number of homeostatic functions in the body (Fujita, 1988). The current investigation revealed that dysfunction of many other organs may be due to thyroid malfunction (Dimitriadiis et al., 1991; Hopasen et al., 1989). But the mechanism of thyroidal effects on other organs in the body is still imprecise. We hypothesized thyroid hormone may have an important role in maintaining the cytomorphology of retina of eye and bicep muscle. Although it has been recognized for many years that symptoms and signs indicating disordered function of the voluntary musculature may be prominent in patients

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# All are equal contributors
with myxoe dema, the muscular manifestations of hypothyroidism have received comparatively less attention. Kocher (1892) reported that patients with sporadic cretinism or myxoe dema often showed bulky musculature, diminished muscular power, and sluggish movement with or without "tetany". Hoffmann (1897) later described features suggestive of myotonia in a patient who had undergone thyroide catomy several times; he pointed out that in this case there was a striking delay in muscular relaxation, reminiscent of that which occurs in myotonia congenita (Thomsen's disease).

In fact, however, the abnormality of muscle function which results from hypothyroidism must be distinguished from true myotonia, as the overall slowness of muscular activity affects muscular contraction as well as relaxation and the typical electrical after-discharge of myotonia is not seen.

Hence the term "pseudomyotonia" is a more appropriate description for the myxoedematous phenomenon. Thyroxine also act on eye tissue through receptor in orbital fibroblasts and in normal orbital-adipose cells (Heufelder et al., 1993). Therefore, we induced hypothyroidism with the treatment of methimazole in rats to understand this complex interaction. We also investigated the histological changes of retinal tissue and biceps muscle.

**MATERIALS AND METHODS**

The laboratory experiments were performed using rat (Rattus rattus). Normal young adult rat aged 8-10 weeks and weight 100-110 g were housed in polypropylene cages and were acclimatized in laboratory condition for a week with natural light and dark schedules prior to experimentation.

The animals were fed standard rodent diet and water was provided ad libitum. They were divided into hypothyroid and their respective control groups. Group I animals were treated with Methimazole (Sigma, USA) 20 mg/kg body weight/day for 14 days. Before autopsy, rats were anesthetized with chloroform. Blood was drawn directly from the heart for serum T₃, T₄ hormones.

Eye tissues and biceps were dissected out and fixed in Bouin’s fixative (Parakkal, 1961). Tissues were embedded in paraffin, sectioned (5 μm), mounted on glass slide and stained with hematoxylin-eosin for histological analysis. Retinal tissues were also stained with Masson’s Trichrome for collagen study. T₃ or T₄ concentrations in rat serum were determined by radioimmunoassay (RIA).

**RESULTS**

**Serum T₃ and T₄ level**

The results showed significant changes in T₃, T₄ hormones (Figure 1A and B). The serum T₃ and T₄ levels were significantly reduced in hypothyroid rat confirming that they were indeed in a hypothyroid state.
Hypothyroidism Causes Cellular Damage in Retinal Tissue and Bicep in Rat

Figure 1. Plasma concentrations (ng/dL) of T3 (A) and T4 (B) in hypothyroid and control rats. Values are expressed as Mean ± SEM.

**HISTOLOGY OF RETINA**

Hypothyroid rats exhibited marked changes in the general cytomorphology of retina. A significant number of cells were found to be reduced in outer and inner nuclear layer in hypothyroid group. The outer and inner nuclear layer lost their individual boundary (Figure 2A and B). The amount of collagen fibres were reduced in retinal tissue in hypothyroid rat (Figure 3A and B).

Figure 2. (A) Normal rat retina(x 400) (B) Hypothyroid rat retina(x 400).
HISTOLOGY OF BICEPS

Important changes were observed in the histology of biceps in the hypothyroid group when compared with the control group under light microscope (Figure 4A and B). Hypothyroid rat exhibited degenerated muscle fibres. Normal biceps muscle showed relatively uniform fiber diameter, peripherally located nuclei, with no fiber degeneration (4A). By contrast, the hypothyroid sections showed variation in fiber diameter, distinctive perifascicular fiber atrophy (4B). The existence of grouped atrophy and pyknotic nuclear clump in muscular fibres suggest muscular degeneration (4C).

Figure 3. (A) Collagen in normal rat retina(x 400) (B) Collagen in hypothyroid rat retina(x 400).

Figure 4. (A) Control rat bicep(x 400) 4 (B) hypo rat bicep(x 400) (variation in fiber diameter).
DISCUSSION

In the present investigation, we used methimazole to induce hypothyroidism as reflected in the serum T3, T4 levels (Pantos et al., 2005; Grofte et al., 1997). It has been previously established that the clinical symptoms of thyroid eye disease (TED) is associated with hyperthyroidism (Prummel et al., 1990). In the case of thyroid eye disease, the damage is directed to fatty tissue behind the eye. Swelling of the damaged tissues behind the eyes can cause the eyes to become red.

It may also cause the eyes to be pushed forward (‘starey eyes’, ‘proptosis’). Occasionally, the swelling behind the eyes is bad enough to press on the nerve from the eyes to the brain affecting vision (Kendler et al., 1993). Graves’ disease is the only kind of hyperthyroidism that has inflammation of the eyes, swelling of the tissues around the eyes, and bulging of the eyes (called Graves’ ophthalmopathy). It is almost always associated with Graves' disease (GD) but may rarely be seen in Hashimoto's thyroiditis, primary hypothyroidism, or thyroid cancer. But in our result we found changes in the histology of retina after induction of hypothyroidism.

Number of cells became reduced in outer and inner nuclear layer in hypothyroid group. The outer and inner nuclear layer lost their individual boundary. The amount of collagen fibres were reduced in retinal tissue in hypothyroid rat.

Our result also showed the changes of cytomorphology in biceps of hypothyroid rats. Hypothyroid sections showed variation in fiber diameter, distinctive fiber atrophy. The existence of grouped atrophy and pyknotic nuclear clump in muscular fibres suggest muscular degeneration. Hypothyroid myopathy has so far been reported in long standing cases of hypothyroidism (Astrom et al., 1961; Wilson et al., 1959). It presents with severe muscle aches and cramps, stiffness and spasms (McKeran et al., 1975, 1979). Histologically, myofibrillar degeneration is present. The atrophic form may resemble the more familiar muscular dystrophies. Therefore, hypothyroidism may be an important cause for visual problem as well as thyroid myopathy.

Figure 4. (C) Hypothyroid rat biceps(x 400) (existence of grouped atrophy and pyknotic nuclear clump in muscular fibres).
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ABSTRACT

The American Visceral Leishmaniasis (VL) is a serious chronic disease, potentially fatal to humans; its lethality can reach 10% if no proper treatment is used. The growing urbanization of the disease occurred in the past 20 years and puts the discussion on control strategies because Brazil is considered a major outbreak of the disease, which originated approximately 90% of the cases in the Americas. In recent years, the lethality of VL is increasing gradually, pointing to infectious complications and bleeding as the major factors of risk for death. In this sense, this work aims a revision of the Brazilian historic of the disease, infection ways, symptoms and fatality rates since it was discovery in Brazil.

Descriptors: American visceral Leishmaniasis, VL and Leishmaniasis

INTRODUCTION

American visceral leishmaniasis (VL) is a generalized infection of mononuclear phagocytic system (MPS) – Mobile Defense System – caused by protozoa of the gender Leishmania subgener Leishmania, but only Leishmania (Leishmania) infantum chagasi causes visceral leishmaniasis, and the only present in the Americas [1,2]. The worldwide incidence of leishmaniasis is estimated in 2 million new cases per year, these numbers are...
probably underestimated, since it is assumed that for every one case diagnosed in the Americas, five more were not identified [3].

According to Desjeux (2001) [4], migration, human pressure, urbanization of the cycles of circulation of *Leishmania*, immunosuppression and malnutrition of the host are considered as risk factors that would explain the expansion, the spread and increase of incidence of leishmaniasis cases in many regions of the world. Roger, 1908 cited in Rey, 2001 [5] demonstrated that the earliest references of VL are from 1882, when it was described a special form of tropical splenomegaly, which occurred endemically in parts of India, associated then with hookworm or a special type of malaria.

VL has a wide distribution in the Old and New World, located among one of the seven endemics priority attention of World Health Organization. VL affects 500000 people each year and approximately 90% of all cases reported worldwide come from: Bangladesh, Brazil, India, Nepal and Sudan. The disease affects primarily the poorest countries, associated with malnutrition, sanitation and disorderly occupation of the environment. In the new world this disease is known as kalazar or neo-tropical American visceral leishmaniasis [3].

In Brazil, the first known case was diagnosed in 1913 by Migone [6, 7], it was a clinical case of an Italian immigrant who, after living 13 years in Santos, travelled to Mato Grosso where he got ill [6]. In 1936, at a commission headed by researcher Evandro Chagas, who investigated the occurrence of several cases of VL, Deane and Mangabeira [7] incriminated *Lutzomyia longipalpis* mosquito as the transmisor, and in 1937, Chagas found the first case of canine VL in Ceará, Castro and Ferreira [1, 6].

Until 1953, there was only 43 confirmed cases of VL in vivo had been reported against 332 cases found by visceroctomy by Madureira Pará [7], distributed in the states of Ceará, Bahia, Sergipe, Pará, Piauí, Rio Grande do Norte, Pernambuco, Mato Grosso, Maranhão, Paraíba and Minas Gerais [1].

In 1953, began to emerge many cases of the disease, especially in Ceará. In 1954, Deane and Deane [8] incriminated the Fox *Lycalopex vetulus* as the wild reservoir in Brazil [1]. Other reservoirs, in addition to *L. vetulus*, are also pointed as the man, the fox *Cerdocyon thous* (wild reservoir), and the dog *Canis familiaris* (domestic reservoir) [9].

*L. longipalpis* is the only vector species with epidemiological importance involves in the disease transmission [2]. The transmission mechanism to humans is through the bite of the sandfly, which inoculates the agent on the surface of the dermis where it multiplies in the cytoplasm of local FMS cells.

It spreads by hematogenous route, reaching greater intensity the viscera with a bigger number of FMS cells: liver, spleen, bone marrow and lymph nodes. Other systems are also infected such as the digestive tract, lungs, skin, blood vessels and kidneys, with nephropathy related to immune mechanisms, once this condition has been included in the group of autoimmune diseases [1, 10].

The diversity of vectors and reservoirs is large due to the extent of the endemic region and the fact that the disease often occurs in focus separated by geoclimatic barriers [2]. The sandflies easily colonize the canyons, mountains, foothill of mountains, in addition to the interior of river valleys, having to high hability to adapt temperatures and low degree of humidity, which is the period of greatest transmissibility in the rain season. VL especially in a rural endemic disease, but also affecting the urban periphery, affects about 3000 people every year in Brazil [1, 9, 10]. Accordingly, the present study presents a review on the Brazil’l’s history of VL since its discovery until the means of infection, symptoms and death rates.
HISTORICAL EPIDEMIOLOGICAL ASPECTS OF AMERICAN VISCERAL LEISHMANIASIS

EPIDEMIOLOGY

VL is a parasitic disease found in rural areas of almost all tropical and subtropical regions at the global [11, 12, 13, 14]. The epidemiology of visceral leishmaniasis in the world varies largely according to the *Leishmania* species causing the infection and the vector species involved in transmission. VL can be divided in didactic terms in two large clinical epidemiological groups: VL caused by *L. (L.) infantum* and *L. (L.) chagasi* (considered as the same species) and VL caused by *L. (L.) donovani* [2].

The infection caused by *L. (L.) chagasi* is restricted to the Americas. Despite this extensive endemic area, *Lutzomyia longipalpis* is the only vector involved in the disease transmission, and the dog the main reservoir [1, 2, 13, 15, 16]. Deane *et al.* [7] demonstrated that 24.8% of sandflies become infected by biting an infected dog whereas to 14.8% of them became infected by biting a human. Therefore, canine VL is more prevalent than human and above the endemic [1]. The first study reporting canine infection in the Americas was published in Brazil in 1930 by Evandro Chagas [17] who found 4.1% of infected dogs in the towns of Moju and Abaetetuba in the Pará state. On the other hand the first reservoir to be described was the fox *Lycalopex vetulus*, a common canid frequent in rural areas of Brazil. This is an important reservoir due to its intense parasitism, higher than the dog, as to be responsible to the spread of *E. hystolytica* infection to distant sited due to migratory habits [1]. Other animals were also reported, including the fox *Cerdocyon thous* and some marsupials, *Didelphis marsupialis* and *Didelphis albiventris* [2, 9].

VL has a wide distribution in our continent, but Brazil represents the mains focus, with 90% of the reported cases in the Americas (6). One worrying aspect of VL epidemiology in Brazil is the urbanization of the disease that has expanded its areas of transmission to the suburb of large urban centers as shown in outbreaks in Teresina, São Luiz, Fortaleza, Camaçari-BA, Montes Claros, Minas Gerais, Belo Horizonte and Rio de Janeiro [2, 11, 12]. The main focuses of this parasitosis in Brazil are located in semi-arid and arid regions, particularly in Northeast and part of Southeast, and the transmission is observed in the foothills of mountains and ditches, rocky terrain with caves and creeping vegetation (shrubs). In Minas Gerais the highest prevalence is observed in the Drought Polygon (Montes Claros, Januária) and Vale do Rio Doce – São Francisco and Jequitinhonha [11]. In these areas, the local ecological factors, such as hot, humid climate and abundant vegetation in shrub are favorable factors for the vector [6].

In Pernambuco, were reported 336 LVL cases, during the period of 1934 to 1984, 51.78% were found in an area from hinterland region (Araripina, Salgueiro) and 31.25% were concentrated in the metropolitan area of Recife and a small region of Deciduous Forest. In the 90s, about 90% of the reported cases of VL occurred in the Northeast. However, in the period of 2000-2002, the Northeast has showed a reduction of 77% of the reported cases in the country [1, 6, 11, 18].

According to the Brazilian Ministry of Health in 19 years of notification (1984-2002), 48455 cases of VL have been reported, with approximately 66% of them occurring in the states of Bahia, Ceará, Maranhão and Piauí [18]. Over the past ten years about 3000 cases per year have been registered in the country, with an annual average of 3156 cases (Figure 1) [20]. In the period of 2000 to 2008, there was an average of around 200 deaths annually, with a mortality rate of 6.5% (Figure 2) [21].
The spread of endemic areas and the emergence of new focus are caused by changes in the environment, which are caused by intense migratory process due social or economic pressures, the poverty, the rising of urbanization process, the emptying of countryside and periodic droughts. This leads to a reduction in the ecological space of the disease, facilitating the occurrence of epidemics [18].
RESERVOIR

In urban areas the dog (*Canis familiaris*) is the main source of VL infection, being the domestic reservoir, responsible for maintaining the disease cycle in the home environment [18]. Once infected, the dog presents intense cutaneous parasitism by *L. (L.) chagasi*, which makes it an excellent source of infection for the sandfly vector. The enzootic canine has preceded the occurrence of human cases, and infection in dogs has been more prevalent then in man. However, like the man, the dog is just an accidental host of the parasite. In sylvatic environment, reservoir is foxes (*Dusicyon vetulus, Cerdoyon thous* and *Lycalopex vetulus*) and marsupials (*Didelpis albiventris*) [9, 18]. The fox is the primitive wild reservoir of VL, in which the infection by *L. (L.) chagasi* causes no harm to its health of this animal [6, 19]. In Brazil, the foxes were found infected in the Northeast, Southeast and Amazon [6]. Marsupials didelphids were found infected in Brazil and Colombia [1, 11, 15, 16].

VECTORS

The vectors of VL are insects known as sand flies, popularly known as straw mosquito, tatuquira, birigui, among others. In Brazil, so far two species have been related to the disease transmission, *Lutzomyia longipalpis* and *Lutzomyia cruzi*. The first reported is considered the main transmitter of *L. (L.) chagasi* in Brazil and the latter has been reported as a vector in the State of Mato Grosso do Sul [6, 11, 18].

In Brazil, the geographic distribution of *L. longipalpis* is broad and seems to be expanding. This species are found in four geographic regions: Northeast, North, Southeast and Midwest. In the North and Northeast, *L. longipalpis* was originally found in the forest participating in the primary cycle of disease transmission. Gradually the insect has adapted to the rural environment. At the end of the 80s, there was an adaptation of this vector in urban areas, on the suburbs of big cities, mainly in the Southeast, can be found in animals sheds, henhouse, kennel, barns and other environments and in home [18].

These insects are small, measuring 1-3 mm in length. They have their body covered by hair and are light colored (ten or straw color). They are easily identified by their behavior, when flying in little jumps with wings half open. These insects, in adulthood, are adapted to different environments, but during larval it develops in moist environments, rich in organic matter and low light incidence. Both genders need carbohydrates as energy source and the females feed on blood for egg development [18].

The life cycle of *L. longipalpis* includes four phases: egg, larva (with four stages), pupa and adult. After copulation the female lay their eggs on moist soil with high organic material content, to ensure the feeding of the larvae. The eggs usually hatch 7 to 10 days after laying. Larvae develop between 20 and 30 days, according to environment conditions and adverse conditions the fourth stage can enter in diapauses. After this period the fourth stage larvae turn into pupae, in this period they are more resistant to changes in humidity than the eggs and larvae. The pupae do not feed and have air breathing. The pupal period under favorable conditions lasts on average one to two weeks [18].

The egg development to adult insect takes a period of about 30 to 40 days according to the temperature. Females feed on blood mandatory, present eclectic habits and can feed on
blood of several species of vertebrates, including humans. The longevity of females is estimated in around 20 days [18].

The sandflies are active during twilight and night. During the day, these insects are at rest in humid places, protected from wind and predators. The infection of vector occurs when the females feed on blood of infected mammals, ingesting macrophages infected by amastigotes forms of *Leishmania*. In the anterior digestive tract occurs the first disruption of macrophages releasing these amastigotes. They multiply by binary division and differentiate rapidly into flagellated forms named promastigotes, which also reproduce through successive processes of binary division. The promastigotes develop into paramastigotes which colonize the pharynx and esophagus of the vector, which remain adhered to the epithelium by the scourage, when they differentiate into promastigote metacyclic infective form. The cycle of the parasite in the insect is completed around 72 hours [18].

The infective females release the metacyclic promastigote forms in her saliva during the next feed on a vertebrate. In the epidermis of the host, these forms are phagocytosed by immune cells in the vacuole of macrophages. The parasite differentiate into amastigotes and multiply intensively until breaking off the cell, releasing these forms to be phagocytosed again by others macrophages in a new continuous process, occurring then the spread by the blood to other tissues, such as lymph nodes, liver, spleen and bone marrow [18].

**TRANSMISSION MODE**

In Brazil, the mode of transmission is through the bite of the sandfly vector, *L. longipalpis* or *L. cruzi* infected by *Leishmania (L.) chagasi* [12, 19, 22].

There is no evidence of a man to man transmission. Therefore, the majority of human infections would be acquired by the bites of sandflies that have previously bitten infected dogs. It was demonstrated experimentally that the sandfly can transmit the disease 7 days after feeding on infected blood [6, 18, 22].

And that transmission occurs while there is parasitism in the skin or peripheral blood of the host. There are described cases of congenital transmission (six cases), sexual intercourse, blood transfusion or laboratory accident [11, 12, 18, 19, 22].

It is not evident whether there is a certain time of year especially, in which the transmission rate is higher among dogs and people. The main reason is that the exact date of infection cannot be determined. However, there is evidence that the period of highest transmission of VL occurs right after the rainy season, when an increased in insect population density happens [6, 18].

**INFECTION**

When the infected vector bites a person, the peptides responsible for the inflammatory action attract phagocytic cells to the site of the bite, and the promastigotes penetrate these cells, transforming into amastigotes, escaping the first nonspecific defense mechanism such as lysis by the complement system. From this point on most people can contain the infection (unapparent infection) and establish a lasting immunity. Others develop an oligosymptomatic form of infection that can also resolve spontaneously or develop into the classic disease. An
unknown percentage of individual, probably most, develops immunity to reinfection, but without eradicating the parasite from the body, which may again multiply in situations of immunodeficiency [9, 18].

In the individuals who progresses of the disease, the infected macrophages burst releasing the amastigotes forms which leads to invasion of new macrophages. Infected macrophages produce a chemotaxins that will attract new macrophages that become infected creating a vicious cycle that will culminate in the exponential multiplication of the parasite [2].

Unapparent or asymptomatic infections are those in which there is no evidence of clinical manifestations. Diagnosis is done by serological or Montenegro tests or antibody titers are generally low and may remain positive for a longer period. It is important to point out that patients with clinical cure or with cutaneous leishmaniasis (cutaneous and mucosal forms) may present reactivity in serological and Montenegro tests. Therefore, the asymptomatic forms are observed in patients from endemic areas, where there is epidemiological and immunological evidence of infection [18].

SYMPTOMS

The main symptoms and signs of VL include prolonged irregular fever, hepatosplenomegaly, pancytopenia, weight loss, malnutrition, immunosuppression, cachexia, stomatitis, skin peeling, earthy pale, pellagra dermatitis and angular cheilosis [1, 6, 9, 12, 16, 22]. There is a decreased function of mononuclear phagocytic system (MPS) organs, on the site of parasite multiplication that suffers hyperplasia and hypertrophy in response to the infection [2]. It is possible to observe organic structural or tissue changes such as splenomegaly, hepatomegaly, adenopathy, anemia, leukopenia and increase of serum gammablobulin [11, 22].

The period of infection is difficult to be determined because it may vary from three to six months [1]. The skin may show a small ulceration, which may go unnoticed or a small granuloma at the site of insect bite and is constituted by histiocytes filled with amastigotes cells surrounded by epithelial cells followed by giant cells [11].

After 120 to 180 days, there is dissemination of parasites, primarily in the lymph nodes and then through blood to the organs [11]. The lymph nodes are presented systematically and moderate with infarction, predominantly presenting inguinal and axillary micropoliadenias. It is possible to see myeloid transformation of medullar cords and numerous histiocytes rich in amastigote cells [1, 11]. In the bone marrow occurs a progressive substitution of hemopoietic tissue and the fat mass for cells of MPS infected with amastigotes. Initially occurs a hyperplasia of endothelial reticule, until developing into a bone marrow failure, it also occurs a loss of hemopoietic function in precursor cell of plasmocyte due to the production of bloodline cells [2, 11]. The lungs are enlarged and congested. Interaveolar septa are thickened due to inflammatory infiltrate consisting mainly of lymphocytes, macrophages and plamocytes. Interstitial cells are increased because of the presence of fat vacuoles, mild edema with congestion of septal capillaries [2]. About 80% cases present interstitial pneumonitis, with dry and unproductive cough, steroids grunts and bronchitis [1, 2, 11].

In the digestive tract, especially in the small intestine, there is proliferation of infected MPS cells by amastigotes forms, located at the duodenal-jejunal microvilli. The jejuna injury is characterized by inflammatory infiltrate composed of heavily parasite macrophages,
plamocytes and lymphocytes; with an atrophy of crypts cells and loss of albumin responsible for the low plasma levels if this protein in the development of the disease and decrease of intestinal absorption may occur small ulceration in presence of the parasite. Also there is edema, enlargement of the villi and intestinal lymphatic vase, without changes on the structure of the organ. The appetite is normal or often increased [1, 2, 11].

The liver is always enlarged, with the surface slightly yellowed due to steatosis, it presents lightly hardened and with no nodules and can be felt up to 15 centimeters below the costal margin [1, 2, 11]. It can evolve a long-term liver fibrosis that compromises the permeability of sinusoidal wall. Also occurs hyperplasia and hypertrophy of Kupffer cells, which contain the parasite [2, 11]. Kidneys have interstitial nephritis, pinpoint hemorrhages, an interstitial inflammatory infiltrate because of macrophages and plasmocytes mainly around the vessels of renal cortex. The parasites are rare and found in the endothelium of glomerular capillaries. In chronic cases there are deposits of immunocomplexes in basement membrane of glomerulus and in fatal cases occur in hyaline thickening in glomerular mesangium [2,11].

Visceral leishmaniasis in the spleen causes severe splenomegaly. Its increase is due to hyperplasia of the MPS and large amounts of amastigotes cells within the splenic pulp. There is a decrease in the number of T lymphocytes mainly in the T dependent areas [2, 11].

At the end of the first month the spleen reaches beyond the costal margin, may get to the lower abdomen [1]. The Malpighian corpuscles are sometimes hypertrophied, sometimes are atrophied, the follicular arterioles are thickened, its endothelium is full of parasites and the organ is congested. Although there is an increase of connective tissue, hyaline degeneration, fibrous nodules and granulomatous Gandygama type [11]. In the acute phase of infection, the capsule of the organ is smooth, soft and often its weight caries between 500 g and 2.0 kg. In the chronic phase the organ is hard, firm and painless to palpation, there is a process of fibrosis, atrophy of lymphoid follicles, myeloid metaplasia, and can occur anemic infarcts [11]. Anemia is responsible for the pale and its progressively worsening may lead to cardiorespiratory manifestations with syncope episodes, anemic cardiac murmur [11]. If leishmaniasis is not treated properly it can develop an aggravation of the disease as extreme cachexia, severe muscle hypertrophy, depletion, massive hepatosplenomegaly, severe hemorrhagic manifestations, delirium, hallucinations and infectious complications that generally leads to death [1].

In the last two decades, VL reappeared in the world to an alarming extent. In Brazil, urban epidemics were observed in several cities and the disease has been established as an opportunistic infection in patients with AIDS, as has been observed in the south of Europe [23]. Moreover, the expansion of the epidemic affecting young people or groups of individual with co-morbidities has caused high number of deaths.

The infectious complications and hemorrhage are the main risk factors to lead to death in VL. Early diagnosis is crucial to reduce mortality through the introduction of effective prophylactic and therapeutic measures [23].

**FINAL CONSIDERATIONS**

According to literature review, it is possible to see the need for an effective program to control the disease, so that we can avoid the risks of disease transmission and take prevention measures. For such health programs, efforts are needed from the population and government,
especially regarding sanitation, control of dog populations, vaccine against canine VL, early diagnosis, among many other preventive measures. Only then we could think of eradicating VL from our country.

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HYPOTHYROIDISM REDUCES PHAGOCYTIC ACTIVITY OF RAT MACROPHAGE

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ABSTRACT

Thyroid dysfunction may have an important role in immune response in mammal. We aimed to understand the role of thyroid hormones in peritoneal macrophages activity in rat. Methimazole induced hypothyroid rat exhibited significant morphological changes in peritoneal macrophages.

The phagocytic activity of peritoneal macrophages was reduced in hypothyroid group. Consequently hypothyroidism induced apoptosis of the macrophages. Therefore, thyroid dysfunction may be an important cause for immune dysfunction in mammal.

Keywords: Thyroid, methimazole, thyroxine, macrophage, phagocytosis, hypothyroidism

INTRODUCTION

Thyroid hormones promote normal growth and development and regulate a number of homeostatic functions in the body (Fujita, 1988). In recent times, thyroid diseases are being diagnosed very common and its relation with immune modulation has been the topic of intense investigation.

Hypothyroidism may be an important cause of altered macrophage function in mammal (Liu et al., 1993; Schoenfeld et al., 1995).

Our study investigated the changes of peritoneal macrophage phagocytic activity in rat under experimentally induced hypo and hyperthyroid condition.

The observation from the study may help to understand how thyroid dysfunction is correlated with immune disorder.

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MATERIALS AND METHODS

Animals and Housing

The laboratory experiments were performed using rat (Rattus rattus) as a mammalian model. Normal young adult rat aged 8-10 weeks and weight 100-110 g were housed in polypropylene cages and were acclimatized in laboratory condition for a week with natural light and dark schedules prior to experimentation. The animals were fed standard rodent diet and water was provided ad libitum.

Treatment of Animals

Rats were divided into hypothyroid, hyperthyroid and their respective control groups. Group I animals were treated with methimazole 20 mg/kg body weight/day for 14 days. Group II animals were treated with thyroxine 600 µg/kg body weight/day for 14 days.

Macrophage Isolation or Extraction

Activated charcoal particles suspended in normal saline (0·9% NaCl) or cold 0·1 M phosphate buffer saline (PBS, pH 7·2) was injected into rat peritoneum and the aspirate was taken for macrophage study (after Iyengar et al., 1985).

Fixation and Staining of Macrophages

Peritoneal fluid was placed and smeared directly on sterilized glass slides and incubated at 37°C in a humid chamber for 3 hours. After incubation the nonadherent cells were removed by washing three times with 0·9% NaCl.

The adherent macrophages were fixed by methanol. After fixation macrophages were stained by Giemsa and observed under light microscope (after Iyengar et al., 1985). Different stages of phagocytosis of charcoal particles by peritoneal macrophages on glass slides were determined in both control and treated rats. Macrophage morphology was examined by hematoxylin-eosin staining.

Fluorescence Microscopic Study of Macrophages

Macrophage destruction was determined by nuclear DNA fragmentation by using DAPI under fluorescence microscope.

Counting of Macrophages

We used peritoneal aspirate for cell counting by hemacytometer.
Thyroid Hormones (T3 and T4) Assay

Rats were anesthetized with chloroform. Blood was drawn directly from heart for serum T3, T4 hormones. T3 or T4 concentrations in rat serum were determined by radioimmunoassay (RIA).

Statistical Analysis

All the data were presented as mean ±SEM. The data were analyzed by t-test. Results with P < 0.05, P< 0.01, P< 0.001 were considered statistically significant.

RESULT

Serum T3, T4 Level

The results showed significant changes in T3, T4 hormones (Figure 1A and B). The serum T3 and T4 levels were significantly reduced in hypothyroid rat whereas in contrast the levels significantly elevated in hyperthyroid rat as expected and confirming that they were indeed in a hypothyroid and hyperthyroid state.

![Figure 1. Plasma concentrations (ng/dL) of T3 (A) and T4 (B) in hypothyroid and hyperthyroid rats. Values are expressed as Mean ± SE.](image)

Giemsa Staining of Macrophages

Significant changes were observed in the cytomorphology of peritoneal macrophages in the hypothyroid group when compared with the control group under light microscopy (Figure 2A and B). Hypothyroid rat exhibited cellular damage. A significant percentage of peritoneal...
macrophages become picnotic in hypothyroidism. Normal macrophages showed different stages of phagocytosis. But the necrotic macrophages in hypothyroid group were not able to phagocytose the charcoal particles. No discernible change in peritoneal macrophages was detected in hyperthyroid rats (picture not shown).

Figure 2. (A) Different stages of phagocytosis in normal rat peritoneal macrophage, A1-A2: movement of macrophage towards charcoal particles, A3: attachment of charcoal particle on macrophage surface, A4: phagocytosis of charcoal by macrophages. (B) Apoptotic peritoneal macrophages in hypothyroid rat.

Figure 3. (A, B): A) Normal rat peritoneal macrophage (x 400), (B) Hypothyroid rat peritoneal macrophage (x 400).
Eosin-Haematoxylin Staining of Macrophages

General morphology of macrophage was altered in hypothyroid rat. Significant number of hypothyroid rat peritoneal macrophages showed membrane blebbing (Figure 3A and B).

DAPI Staining of Macrophages

Hypothyroid macrophages showed different stages of nuclear fragmentation (Figure 4A and B). DAPI stained all the nuclei blue.

Macrophage Counting

Significant number of macrophages was reduced in hypothyroid group. (13.0 ± 1.7 vs. 1.9 ± 1.3 macrophages /mm², P < 0.05; Figure 5)
**DISCUSSION**

In the present investigation, we used methimazole to induce hypothyroidism and thyroxine to induce hyperthyroidism, as reflected in the serum T3, T4 levels (Pantos et al., 2005; Grofte et al., 1997). The present study clearly indicates that hypothyroidism adversely affects peritoneal macrophages function. Thyroid hormones are essential for normal function of macrophages. Their effect may be either direct or indirect (Liu et al., 1993; Rosa et al., 1995). In hypothyroidism a significant number of peritoneal macrophages were found to be picnotic. The number of peritoneal macrophages reduced in hypothyroid rat. The reason for the reduction of cell number after methimazole treatment is apparently due to an increased rate of peritoneal cell apoptosis (Liu et al., 1993)

An increased rate of macrophage death due to apoptosis causes a decrease of immune function in hypothyroidism (Liu et al., 1993; Schoenfeld et al., 1995). The DAPI stained hypothyroid peritoneal macrophages showed different stages of nuclear fragmentation indicating apoptosis. The cellular death was also confirmed by membrane blebbing. The previous histological studies revealed that methimazole induced hypothyroidism caused cell damage. This damage is associated with an increase of oxidative stress by producing ROS. In methimazole induced hypothyroidism catalase activity is reduced and this allows H2O2caused damage (Cano-Europa E et al., 2010). The phagocytic behavior of macrophages was altered in hypothyroid group. The resulting reduction and death of peritoneal macrophages in hypothyroid rat can provide an explanation for the decrease of immune responses and phagocytosis in hypothyroidism (Liu et al., 1993; Schoenfeld et al., 1995).

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LIFE CYCLE AND GROWTH RATES OF THE CONICAL SNAIL COCHLICELLA ACUTA (MÜLLER, 1774) (GASTROPODA: COCHLICELLIDAE)

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ABSTRACT

Mating, pre-oviposition, oviposition, post-oviposition, and generation periods as well as growth rate of the pointed or conical snail Cochlicella acuta (Müller, 1774) were studied in Egypt under laboratory conditions during its reproductive and activity season of 2008/2009. In spite of this species is hermaphroditic, the laboratory observations indicated that cross mating was essential for oviposition as unmated snails did not lay any egg. Mating occurs when snail individuals reach their sexual maturity as calculated from adulthood till mating. This period was 99.2 ± 23.8 days in average. Pre-oviposition period (from mating till laying first egg) lasted for an average of 17.6 ± 6.1 days. Oviposition period (from laying first egg till last one) averaged 86.3 ± 22.5 days during which snail individual deposited a total of 113.3 ± 58.3 eggs. Post-oviposition period (from last laid egg till mortality) was 140.7 ± 74.3 days. The generation period (from egg to egg) of this species extended to an average of 283.7 ± 29.3 days.

Keywords: Cochlicella acuta - Sexual maturity – Oviposition – Generation period – Growth rates

INTRODUCTION

The pointed or conical snail Cochlicella acuta (Müller, 1774) is a native of coastal areas of the Mediterranean and Western Europe (Lewis, 1977; Kerney and Cameron, 1979). However, Baker (1986) stated that this species is an introduced agricultural pest in south eastern and south Western Australia. He added that large numbers of C. acuta aestivate in Australia on the ears and stalks of cereal plants and often clog machinery and contaminate grains during harvest.

Although C. acuta feed on some agricultural plants in the laboratory (Baker, 1989), significant damage to these plants in the field has not been reported.
In Egypt, *C. acuta* is considered one of the most abundant mollusc pest causing damage to palm trees, citrus orchards and ornamental plants. The biology of this pest has been little studied and restricted to non agricultural habitats. The scope of the study reported herein was to examine several biological aspects of the life history (e.g. mating, life cycle, oviposition as well as growth parameters) of *C. acuta* under laboratory conditions.

**MATERIALS AND METHODS**

Seventy adult individuals of *C. acuta* were hand picked from nurseries in Cairo, Egypt, during its active period in November 2008 on different ornamental host plants e.g. rose-scented pelargonium (*Pelargonium gravealens*), night blooming cestrum (*Cestrum noctumum*), ficus trees (*Ficus benjamina*) paper flower (*Bougainvillea glabra*) and golden dewdrop (*Duranta plumieri*).

The snails were kept in three plastic boxes (15 x 7 x 11 cm) containing moist soil to eight centimeters deep and were fed on fresh lettuce leaves (*Lactuca sativa*). The boxes were covered with muslin cloth fixed with rubber bands to prevent the individuals from escaping. Boxes were examined daily. Fresh lettuce leaves and moisture (drops of water) were supplied as required, and the soil was searched for egg clutches.

All egg clutches were removed and placed in prepared pots (13x10 cm) with moist soil and then observed twice daily till hatching to determine the incubation period and hatchability. New hatched snails (juveniles) were placed solitary in plastic cups (13x10cm) with moist soil and fresh lettuce leaves.

The cups were covered to avoid escaping of snails, and were examined daily, where fresh lettuce and moisture were added as required throughout the life span.

Pairs of snails of similar weight and shell diameter were put in prepared pots (13 x 10 cm) and observed several times daily till mating to determine the periods of sexual maturity and copulation.

After that, individuals of every pair were separated and each individual was placed singly in the similar pot to determine the pre-oviposition period, oviposition period, number of egg clutches per individual, clutch size and post-oviposition period.

Juveniles were weighed using a digital balance, and shell diameter was measured using a caliper. This was done monthly from hatching time (zero time) till maturity (according to lips growth). The shell volume was calculated following Baur and Raboud (1988):

Shell volume = \((\text{width})^2 \times \text{height} / 2\)

The growth rate was calculated monthly according to the formula:

Growth rate = \(\frac{W_F - W_I}{W_F} \times 100\)

where \(W_I\) and \(W_F\) are the initial and final weights of snails during a period of month. Also, the rate of change in diameter was calculated using the formula:
Rate of change in diameter \( = \frac{D_F - D_I}{D_F} \times 100 \)

where \( D_I \) and \( D_F \) are the initial and final shell diameter of snails during a period of one month. The rate of change in shell height was calculated as:

Rate of change in height \( = \frac{H_F - H_I}{H_F} \times 100 \)

where \( H_I \) and \( H_F \) are the initial and final shell height of snails during a period of month. Also, the following formula was applied to calculate the rate of change in shell volume.

Rate of change in volume \( = \frac{V_F - V_I}{V_F} \times 100 \)

where \( V_I \) and \( V_F \) are the initial and final shell volume of snails for a period of month. The regression analysis was carried out for each of weight, diameter, height, and volume over age according to the model:

\[ Y = \alpha + \beta X \]

where \( Y \) is the dependent variable (snail weight, diameter, height or volume)

\( \alpha \) is the intercept

\( \beta \) is the slope of regression of \( Y \) over \( X \)

\( X \) is the age in months.

This model was also used to explain the increase and the decrease of weight over one month for the land snails (SAS, 1985).

**RESULTS**

**Mating**

Although this species is hermaphroditic, self-fertilization does not occur in solitary individuals; therefore cross mating is essential to lay eggs. When individuals reached their adulthood according to lip signs, then paired. In *C. acuta* mating occurred when its individuals reached sexual maturity during an average period of 99.2 ± 23.8 days \( (N = 12) \) after adulthood was attained (Table 1). Mating occurred in day time between 10.30 am to 3.30 pm, during its reproductive season (mid November till the end of February).
During copulation two individuals faced each other and attached together strongly from the fleshy parts with a thick layer of mucus for an average period of 142.5 ± 28.1 minutes (N = 6 pairs) (Figure 1). After, individuals gradually pull out their bodies and separated within an average period of 15.0 ± 4.5 minutes.

Table 1. Duration of different stages of *Cochlicella acuta* under laboratory conditions

<table>
<thead>
<tr>
<th>Items</th>
<th>Incubation</th>
<th>Inv.</th>
<th>Life cycle</th>
<th>Sexual maturity</th>
<th>Pre-oviposition</th>
<th>Oviposition</th>
<th>Post-oviposition</th>
<th>Life span</th>
<th>Generation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average ± SD</td>
<td>13.7 ±1.3</td>
<td>251.8±27.5</td>
<td>265.5±27.5</td>
<td>99.2 ±23.8</td>
<td>17.6 ±6.1</td>
<td>86.3 ±22.5</td>
<td>140.7 ±74.3</td>
<td>561.8 ±102.1</td>
<td>283.4±29.3</td>
</tr>
<tr>
<td>Temp. ºC</td>
<td>21.1 ±0.3</td>
<td>24.2</td>
<td>24.0 ±5.1</td>
<td>28.0 ±4.2</td>
<td>20.7 ±1.9</td>
<td>19.7 ±1.5</td>
<td>26.2 ±4.6</td>
<td>25.9 ±4.6</td>
<td>22.4 ±3.8</td>
</tr>
<tr>
<td>R.H.%</td>
<td>48.4 ±7.9</td>
<td>60.1 ±6.8</td>
<td>60.0 ±6.9</td>
<td>57.0 ±7.4</td>
<td>61.5 ±7.4</td>
<td>60.4 ±6.2</td>
<td>60.6 ±6.3</td>
<td>60.5 ±6.3</td>
<td>59.1 ±7.3</td>
</tr>
</tbody>
</table>

Figure 1. Mating process of *Cochlicella acuta*.

**Oviposition**

After mating, individuals of *C. acuta* required a pre-oviposition period averaging 17.6 ± 6.1 days before depositing eggs. Oviposition period (from 1st egg to last one) averaged 86.3 ± 22.5 days during which averages of 13.3 ± 4.1 clutches per snail individual were deposited. The period between two clutches deposit was 6.9 ± 1.7 days. The clutch size ranged from 6.0
to 12.0 with an average of 9.4 ± 2.4 eggs. The total number of eggs deposited by one individual of *C. acuta* during its oviposition period averaged 113.3 ± 58.3 eggs.

Eggs are white, with smooth surface, spherical shape and its diameter ranged from 1.1 to 1.5 mm with an average of 1.3 ± 0.1 mm (N = 21) (Figure 2).

![Figure 2. Eggs of *Cochlicella acuta*.](image)

Laboratory observations showed that both mated individual snails or only one deposited eggs. On some cases both mated snails did not lay any eggs and this might be due to failure in copulation.

**Post-Oviposition**

Post-oviposition period was calculated from last egg deposited till individual death. This period averaged 140.7 ± 74.3 days for *C. acuta*.

**Development**

Egg incubation period of the conical snail *C. acuta* averaged 13.7 ± 1.3 days under laboratory conditions at temperature of 21.2 ± 0.3°C and 48.4 ± 7.9 % relative humidity with a mean hatchability of 64.8 ± 34.8 % (N = 27).

Newly hatching snails (juveniles) are similar to adults but smaller in size and weight. Their average measurements were 1.7 ± 0.3 mm for height and 1.3 ± 0.2 mm for width at zero time (N = 30). They developed slowly in a week activity and reached adulthood after eleven months. At that time their average maximum shell dimensions reached 9.5 ± 1.4 mm for height, 4.3 ± 0.6 mm for width and weighted 0.06 ± 0.012 g (N = 30).

The duration of *C. acuta* life cycle from egg to adulthood (incubation period plus juveniles) averaged 265.5 ± 27.5 days. Mating influenced life span of this species (from egg till mortality). For non-mated, it was 528.1 ± 122.8 days, while it was 561.8 ± 102.1 for mated ones (Table 2).
Table 2. Duration of *Cochlicella acuta* life span in its activity season

<table>
<thead>
<tr>
<th></th>
<th>Mated snails</th>
<th>Unmated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Laid</td>
<td>Non laid</td>
</tr>
<tr>
<td>Average ± SD</td>
<td>561.8 ± 102.1</td>
<td>272.8 ± 194.98</td>
</tr>
<tr>
<td>Range</td>
<td>451 - 705</td>
<td>250 - 455</td>
</tr>
<tr>
<td>N</td>
<td>7</td>
<td>5</td>
</tr>
</tbody>
</table>

Life span of laying snails (life cycle, sexual maturity, pre-oviposition, oviposition and post-oviposition periods) averaged 561.8 ± 102.1 days. On the other hand, this period decreased to half in non-laying snails (life cycle, sexual maturity till death) and averaged 272.8 ± 194.9 days (N = 5).

**Generation Period**

Generation period (from egg to egg) ranged from 237 to 315 days with an average of 283.4 ± 29.3 days (N = 7).

**Snail Growth Performance**

Land snail weight increased with age and reached 0.095 g at five months (Table 3 and 4). Weight decreased gradually to 0.06 g at eleven months of age.

This pattern of weight with age was shown by the significant regression coefficient of 0.004 g per month (Figure 3).

The growth rate of snail shell indicated that early weight gain was highly significant, where the snail was grown by a highly significant rate of 69.68 %, then declined significantly by a rate of 9.14 % each month (Figure 5). The change in snail weight due to growth was also expressed in variable changes in width, height and volume. Shell width steadily increased over age and reached 4.32 mm by the ninth month of age and remained constant during the 10th and 11th months of age. This gradual shell width increase was shown in figure (4), however insignificant regression coefficient of 0.24 was found. The pattern of width change rate was high at early age (26.73%) and insignificantly decreased by 3.63% every month (Figure 4). Shell height was also gradually increased with age to 9.46 mm at nine month of age, with a highly significant regression coefficient of 0.69 mm every month (Figure 4). The change rate in height was highly significant with an intercept of 33.86 mm, indicating that the change occurred in height at early age significantly determined the shell height during life span, although the change rate in height was then regressed insignificantly by 3.65 mm every month (Figure 5). Shell volume was increased from 1.60 mm$^3$ at hatch to 91.49 mm$^3$ at nine months of age. It was indicated that volume was highly significantly increased by 9.0 mm$^3$ every month (Figure 4).
Table 3. Weight, width, height, and volume (Average ± SD) of the land snail *Cochlicella acuta* shell during the life span

<table>
<thead>
<tr>
<th>Age</th>
<th>Average ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Weight</td>
</tr>
<tr>
<td>Hatch time</td>
<td>0</td>
</tr>
<tr>
<td>1st month</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td>2nd month</td>
<td>0.04 ± 0.02</td>
</tr>
<tr>
<td>3rd month</td>
<td>0.08 ± 0.03</td>
</tr>
<tr>
<td>4th month</td>
<td>0.09 ± 0.03</td>
</tr>
<tr>
<td>5th month</td>
<td>0.09 ± 0.02</td>
</tr>
<tr>
<td>6th month</td>
<td>0.09 ± 0.02</td>
</tr>
<tr>
<td>7th month</td>
<td>0.08 ± 0.02</td>
</tr>
<tr>
<td>8th month</td>
<td>0.07 ± 0.01</td>
</tr>
<tr>
<td>9th month</td>
<td>0.07 ± 0.01</td>
</tr>
<tr>
<td>10th month</td>
<td>0.06 ± 0.01</td>
</tr>
<tr>
<td>11th month</td>
<td>0.06 ± 0.01</td>
</tr>
</tbody>
</table>

Table 4. Monthly change rates (Average ± SD) in shell weight, width, height, and volume of the land snail *Cochlicella acuta* over life span

<table>
<thead>
<tr>
<th>Age</th>
<th>Average ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Weight rate</td>
</tr>
<tr>
<td>1st month</td>
<td>100.00</td>
</tr>
<tr>
<td>2nd month</td>
<td>49.8 ± 18.6</td>
</tr>
<tr>
<td>3rd month</td>
<td>51.5 ± 18.5</td>
</tr>
<tr>
<td>4th month</td>
<td>17.9 ± 25.7</td>
</tr>
<tr>
<td>5th month</td>
<td>-3.38 ± 29.1</td>
</tr>
<tr>
<td>6th month</td>
<td>-10.03 ± 15.64</td>
</tr>
<tr>
<td>7th month</td>
<td>-8.04 ± 16.2</td>
</tr>
<tr>
<td>8th month</td>
<td>-11.9 ± 16.8</td>
</tr>
<tr>
<td>9th month</td>
<td>-9.2 ± 12.9</td>
</tr>
<tr>
<td>10th month</td>
<td>-8.8 ± 12.5</td>
</tr>
<tr>
<td>11th month</td>
<td>-5.14 ± 10.2</td>
</tr>
</tbody>
</table>
However the change rate in volume was highly significant throughout the snail shell life span, where it was reduced by 6.88% every month (Figure 5). Significant positive correlation coefficients were found between snail weight rate and volume rate. However, the correlation coefficients between height or width and weight were in general insignificant.

Figure 3. Shell weight of the land snail *Cochlicella acuta*.

Figure 4. Width, height, and volume of *Cochlicella acuta*. 
DISCUSSION

In spite of the conical snail *C. acuta* is hermaphroditic, self-fertilization does not occur, thus cross mating was essential for oviposition as unmated snails did not lay any egg. This result agree with Takeda (1983), Bride and Gomot (1991), Bride *et al.* (1991) and Mohamed and Ali (2009), who stated that egg production in several species of gastropods is stimulated by mating. Also, it was observed that one or both mated snails deposited eggs; it was rarely noticed that both mated snails could not lay eggs and this may be due to copulation failure. These observations are in accordance with Mohamed and Ali (2009) during their studies on the land snails *Monacha cartusiana* (Müller) and *Eobania vermiculata* (Müller). It was noticed that mated individuals of *C. acuta* required an average of 17.6 ± 6.1 days as a pre-oviposition period before depositing eggs.

This agree with Heller (1982) who recorded that *Theba pisana* (Müller) laid its eggs from 9.0 to 14.0 days after copulation and Mohamed and Ali (2009) who mentioned that *M. cartusiana* and *E. vermiculata* laid eggs after 2.7 ± 1.4 and 3.9 ± 1.9 days respectively from copulation.

The results revealed that the growth significance of snail at very early age effect life span thereafter. It was also concluded that the growth of snail shell is significantly explained by weight and volume. However, width and height were both insignificantly contributed to explain the growth pattern of snail life span.
REFERENCES


IMPACT OF NILE TILAPIA CAGE CULTURE ON THE PHYTOPLANKTON COMMUNITY IN A TROPICAL RESERVOIR

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ABSTRACT

Tropical reservoir ecosystems are important for multiple purposes, especially for public potable water supply, agriculture and fish culture. This study investigated the physical-chemical characteristics and the spatial and temporal distribution of phytoplankton community to check alterations in water quality due to impacts caused by cage culture of the exotic fish Nile tilapia, *Oreochromis niloticus* in a reservoir of Northeastern Brazil. The physical-chemical parameters (pH, temperature, electrical conductivity, concentration of dissolved oxygen and light transparency), inorganic nutrients (nitrate, ammonium ions, soluble reactive orthophosphate and total nitrogen and phosphorus) and chlorophyll *a* biomass were measured in two sites of cage culture (point source and non-point source) during two seasons (dry and rainy). Phytoplankton community showed alternating dominance among the species of Cyanobacteria, Bacillariophyceae and Chlorophyceae between the two sites. There was a considerable variation in the levels of inorganic nutrients, such as, nitrate-nitrogen, ammoniacal nitrogen and orthophosphate during the two seasons. The trophic state index diagnosed mesotrophic category, based on the values of chlorophyll *a*, total phosphorus and light transparency. The wind driven turbulence of the water column and the inflow of water (flushing and dilution) during the rainy season acted as constraint which did not permit the luxuriant growth of cyanobacterial species. Cage culture of tilapia did not cause pollution problems which could interfere with the reservoir water quality and the

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cyanobacterial bloom formation. The muted impact was due to the small scale sustained aquaculture in the well managed reservoir.

**Keywords:** Nile tilapia, *Oreochromis niloticus*, cage culture impact, phytoplankton community, Northeastern Brazil

**INTRODUCTION**

The rapid increase in population in tropical regions, and the continued demand for fish, generated the need for inland fish culture as an alternative means of increasing fish production. Nile tilapia, *Oreochromis niloticus* is popularly used for such practices in Brazil. Fish cage culture practices have diverse advantages since they use existing water bodies (reservoirs, rivers and lagoons), low cost economy involved in implementation and the use of simple technology to produce high quality protein and include extension programs of rural development (Chellappa et al., 1995; Chellappa et al., 2011). Another view held explains that this artisan activity helps to clean eutrophicated waters as it involves the culture of planktivorous tilapia species but at the same time an intense practice can cause a series of alterations to the biotic and abiotic components of the environment (Beveridge, 1987).

The universal acceptance is that water quality in lakes and reservoirs is the result of a subtle interaction between physical, chemical, and biological processes acting in spatial and temporal scales. The annual changes in phytoplankton composition and its relative abundance in freshwater aquatic ecosystems, vary from predictable to unpredictable and to chaotic distribution. The mediating factors based on the results of variation in the physical (light and temperature regime) and the chemical (nutrient availability) and these singly or cumulatively stimulate or act as constraints to the phytoplankton growth (Reynolds, 1984; Margalef, 1997; Huisman et al., 2006).

Phytoplankton functional structure and chlorophyll biomass production are often recognized as annual periodicity in species succession or assemblages (Reynolds et al., 2002; Padisák et al., 2003). Light regime experienced by tropical reservoir phytoplankton cells is differentiated into stable period (dry season) and turbulent mixing period (rainy season), which determines the residence time of phytoplankton within the well-illuminated upper layers of the water column (MacIntyre, 1998; Câmara et al., 2009). On the other hand, nutrient distributions and their bioavailability in the upper layers, where light levels are adequate, stimulate better phytoplankton growth.

This is the result of transport processes interacting with biological phenomena. In eutrophic to hypertrophic systems, where nutrients are seldom (if ever) limiting, the zmix/zeu ratio is the main constraint for phytoplankton growth controlling the phytoplankton morphological characteristics and the annual course of the phytoplankton community composition (Naselli-Flores, 2000; Naselli-Flores and Barone, 2007).

Phytoplankton seasonal variation in freshwaters has been successfully applied as functional groups to explain the changes in phytoplankton communities and this principle was based on Reynolds’ model (Reynolds, 1997). It had been related to the response of varying environmental conditions and observed in different freshwater ecosystems viz. temperate lakes (Lindenschmidt and Chorus, 1998), temperate rivers (Descy, 1993), subtropical (Hambright and Zohary, 2000) and tropical ecosystems (Figueroedo and Giani, 2001). In
eutrophic temperate lakes, for example, the dominant phytoplankton functional group identified may change at seasonal scales from group B diatoms to group G chlorophytes, then to group H cyanobacteria and finally to group L dinoflagellates. Phytoplankton communities can also experience changes at sub seasonal time scales, which are driven by short-term changes in the environmental conditions that interrupt, re-set, or alter the phytoplankton community dynamics. Gaedeke and Sommer (1986) demonstrated experimentally that phytoplankton diversity decreased significantly after short-term events of higher turbulence in water, resuspension of sediment materials, strong dilution that led to levels and dissolved nutrients availability. Escot et al. (2004) described the effects of strong wind events on the phytoplankton communities of a Mediterranean reservoir.

Another aspect of phytoplankton ecology was based on anthropogenic effects and the changes of phytoplankton assemblages in favor of bloom formation of toxic cyanobacterial species and considered as negative impact. Recently, Borges et al (2010) investigated the impact of cage culture of tilapia in Rosana Reservoir of the lower stretch of the Paranapanema River in Southern Brazil and observed the dominance of Cyanobacteria. Dominance of *Microcystis*, *Radinocystis* and *Pseudanabaena* has been attributed to the increased predation pressure of zooplankton on other phytoplankton species.

Very few studies have analyzed in detail the impacts caused by fish cage culture practices on the structure and chlorophyll biomass of phytoplankton community. Fish cage culture practice is a key-driver of phytoplankton dynamics in reservoirs to trigger dominant and co-dominant species of cyanobacterial species with consequent fish mortality in the semiarid reservoirs of Northeastern Brazil (Chellappa and Costa, 2003; Chellappa and Chellappa, 2011). The input of organic material, nutrients and metabolites in cage aquaculture environments promote temporary changes in the diet of natural fish populations, especially the opportunists (Strictar-Pereira et al., 2010). It was therefore felt imperative to investigate the changes on phytoplankton community structure and function under the influence of cage culture, an anthropic interference in reservoir environments.

The present study is aimed at two important aspects: firstly, to verify the physical and chemical characteristics of two sites (the point source and non-point source impact of nutrient-energy habitat template of cage culture) and the spatial and temporal variation of phytoplankton community. Secondly, to verify the relative abundance of cyanobacterial species (in the point source and non-point source), besides investigating the tropic status in relation to the effluents of cage culture.

**MATERIALS AND METHODS**

**Study Area**

The present study was carried out in the Reservoir Ministro João Alves, a freshwater body where fish cage-culture is practiced by local fishermen. This reservoir is situated at national grid ref NS latitude 06° 41’ 56” and 06° 41’ 71” S and longitude of 36° 37’ 76” and 36° 370’ 84” W in the city of Parelhas of Northern Brazil. The catchment area has distinctive scrub vegetation, consisting of xerophytic low thorny bushes, dominated essentially by *Acasia* and *Mimosa* spp. adapted to the semi-arid climate. This reservoir was constructed in
1984 on the River Seridó of the Piranhas–Assu hydrographic basin, has a water storage capacity of $10 \times 10^6$ m$^3$ and a theoretical retention time of 780 days (Chellappa et al., 2006; Chellappa et al., 2009a; Chellappa et al., 2009b).

### Table 1. Fish feeding regime used during the cage culture

<table>
<thead>
<tr>
<th>Weight of fish (g)</th>
<th>Feed rate in relation to biomass (%)</th>
<th>Number of feeds per day</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 to 30</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>30 to 100</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>100 to 230</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>230 to 320</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>320 to 420</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Above 420</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

The water outflow is maintained regularly according to the storage capacity and irrigation demands of the region. This area is dominated by humid semiarid climate with high evaporation rate and an irregular pattern of rainfall.

Cage culture of Nile tilapia, *Oreochromis niloticus* is practiced by local fishermen close to the littoral region of the reservoir. The six cages used in the fish culture were made of galvanized iron and wire mesh coated with PVC, plastic drums were used as floats and PVC plastic pipes of 100 mm were sealed at the edges. Each cage measured 2.0 x 2.0 x 1.2 m, with a capacity of 4 m$^3$. Each cage was installed in the reservoir at a distance of 2 meters from each other and cages were stocked with fingerlings of male Nile tilapia, *O. niloticus*, each weighing 1.0 g at a stocking density of 150/m$^3$, totaling to 600 fish fingerlings per cage. The cultivation period was divided into the pre-fattening and the fattening phase. The pre-fattening phase lasted for two months, during which time the fingerlings were offered extruded feed with 42% crude protein (CP). During the fattening phase which lasted for four months, the fish were offered extruded feed with 32% CP. Table 1 shows the feeding regime used during the cage culture.

### Sample Collection and Analyses

Samplings were made fortnightly in two sites: site 1 (the point source) in the vicinity of the cages, and site 2 (the non-point source) which was about 0.5 km from the cages down stream. Samples were collected from the two sites, between 9.00 to 10.00 hrs at fortnightly intervals, from July 2008 to June 2009. Water temperature, pH, dissolved oxygen and electrical conductivity were measured in the field using multiprobe Kit (WTW 340 I–MERCK). Light transparency was assessed using 30 cm Secchi disc. The values of euphotic zone corresponding coefficient of light attenuation were measured by multiplying the Secchi disk depth by 2.7 (Margalef, 1983). Rainfall data was obtained from the Agricultural Research Company of Rio Grande do Norte, Brazil (EMPARN).

Water samples for inorganic nutrients such as, nitrate-nitrogen, ammoniacal-nitrogen and orthophosphate were taken with 2 dm$^3$ Van Dorn bottle and filtered through GF/C fiberglass filters before analyses (Goltermann et al., 1978).
Phytoplankton samples were fixed in Lugol’s iodine solution and the cell counts were done with the help of inverted microscope after 24 hours of sedimentation (Lund et al., 1958). The biovolume procedure based on simple geometric solids was used assuming unit specific gravity (Rott, 1981). The adopted taxonomic classification is based on Hoek et al., (1995). For species identification the manual of Wehr and Sheath (2003) was used. Determination of ecological indices was based on the software programme of Seaby and Henderson (2006). Water samples for chlorophyll a analysis were stored cold at 4°C in the dark and filtered on Whatman GF/C filters. Concentrations were detected after overnight extraction in 90% acetone and corrected from the values of phaeophytin degradation pigment (Marker et al, 1980).

Statistical analyses were performed through Statistics 6.0 software package. Data analysis was based on log-transformed data and subjected to two-way analysis of variance (ANOVA) to test for significance between the dependent (relative abundance of phytoplankton species and independent (environmental parameters) variables.

**RESULTS**

The depth of the reservoir varied dramatically between the rainy and dry seasons during the study period. There was no rainfall during the dry season (July to December) and during the rainy season (January to June) a maximum rainfall of 273 mm was registered. The pattern of rainfall of the region over a period of six years was considered as a trend-setter of the seasons (Figure 1a). It is evident that the rainy season extends from January to June, with shifts in the peak of wet season from year to year. Generally the period of July to December represents the dry season in the semiarid region of Brazil. The mean monthly variation in rainfall during the study period is shown in Figure 1b. Seasonal hydrology of the study reservoir during the period of 2008 through 2009 oscillated according to the dry/wet annual cycle. The rainfall touched a peak of 294 mm during the rainy season and was low as 16 mm in the dry season (Figure 1b). This in turn, largely affected the theoretical renewal time during the period of 2008-2009 which consequently influenced the phytoplankton community.

All environmental variables in sites 1 and 2 during the rainy and dry seasons are shown in Figures 2 and 3. The trend of pH was alkaline both in spatial and temporal scales with a narrow range from 8.3 to 8.6 (Figure 2a). Transparency of water varied from 0.35 to 1.15 cm (Figure 2b) and the temperature varied from 27.2 to 30°C (Figure 2c). During the dry season when water was in a clear state, the mean value of the concentration of dissolved oxygen varied from 6.7 mg L⁻¹ in site 1 to a mean value of 7.3 mg L⁻¹ in site 2. However, this situation was altered during the rainy season, when site 1 reached a maximum of 8.6 mg L⁻¹ and site 2 reached a maximum of 8.0 mg L⁻¹. This coincided with large river influx into the reservoir when the water reached low temperatures (Figure 2d). Oxygen saturation calculated from the temperature and concentration of dissolved oxygen, indicated 80-120% saturation throughout the water column and depletion in oxygen saturation to 65% during the rainy season. The mean electrical conductance ranged from 671–743 µScm⁻¹ during the study period (Figure 2e). These values showed no significant variation during the year, thus maintaining a constancy of 600-700 µScm⁻¹ independent of evaporation during the dry season and from greater allochthonous input from the adjacent catchment areas during the wet season. Mixing
events were frequent during these two seasons of sampling due to wind action and water turbulence.

The values of nitrate-nitrogen (NO$_3$-N) were generally low in comparison to orthophosphate (PO$_4$-P) of the reservoir during the study period. They ranged from 360 in site 0.1 and 0.355 mgL$^{-1}$ in site 2 during dry season to 0.427 to 0.321 mgL$^{-1}$ in site 1 and 2 respectively during the rainy season. Ammonium ion concentrations (NH$_4$-N) were less than nitrate in both sites during the dry and wet seasons, probably due to more oxidation process than reduction. Orthophosphate concentrations varied between 0.570 mgL$^{-1}$ in site 1 and 0.546 mgL$^{-1}$ in site 2 during the dry season to 0.320 in site 1 and 0.409 mgL$^{-1}$ in site 2 during rainy season indicating phosphorus turn over from sediment resuspension (Figure 3).

![Figure 1](image1.png)

**Figure 1.** (a) Rainfall over a period of six years (2004 – 2009); (b) monthly rainfall during the study period.

Chlorophyll $a$ concentrations ranged from 24.0 in site 1 to 11.2 µg L$^{-1}$ in site 2 during the dry season. The levels of chlorophyll $a$ in wet season increased to 29.0 in site 1 and declined to as low as 5.0 µg L$^{-1}$ in site 2 due to turbid water condition and low light penetration (Figure 4).
The clear water phase during the dry months and the larger coefficient of light penetration stimulated more chlorophyll $a$ levels in site 2 of the pelagic zone thus matching with turbid water condition of wet months.

Figure 2. Values of physical-chemical parameters in sites 1 and 2 during the rainy and dry seasons: a) pH, b) transparency, c) temperature, d) concentration of dissolved oxygen, e) electrical conductivity.
Phytoplankton community of the reservoir comprised a total of 42 taxa and this list together with their spatial and temporal distribution is presented in Table 2. A sequence of the abundance of species follows the order of Bacillariophyceae, Chlorophyceae, Cyanobacteria, Dinophyceae, Euglenophyceae and Cryptophyceae for pelagic zone of the reservoir, representing site 2 of non-point source nutrient concentrations. This region was dominated overwhelmingly by species belonging to Bacillariophyceae such as *Aulacoseira granulata*, *Cyclotella meneghiniana* and the chlorophycean alga, *Oocystis lacustris*. This sequence had drastically changed in site 1, where the cyanobacterial species such as, *Anabaena circinalis* with coiled filaments and *A. planktonica* with straight filaments, mucilage colonies of *Microcystis aeruginosa* and very limited *Cylindrospermopsis raciborskii* individuals were
recorded. This was due to the impact from cage-culture of tilapia and a point source of nutrients flux in the site 1 of littoral region. In addition to this, species numbers increased slightly from 17 to 20 taxa. The Dinophyceae was represented principally by species of *Peridinium* and *Gymnodinium* and were numerically reduced.

About 60% of the organisms were with bio volume of less than 1000 $\mu m^3$, even though the dominant species generally showed larger dimensions. The species dominance index expressed high values with filamentous cyanobacterial species in site 1 (impact of cage culture) and two species of bacillariophyceae and one of chlorophyceae in site 2 (Figure 5). Species richness index was used to express the phytoplankton diversity and it showed higher values in site 2 during wet period and synchronized with the appearance of large numbers of chlorococcales species, *Oocystis lacustris*. Species richness index was reduced considerably in site 1 both in wet and dry period due to the expressive dominance of cyanobacterial species (Figure 5).

Table 2. Phytoplankton species registered with their spatial and temporal distribution
in the Reservoir Ministro João Alves, Brazil

<table>
<thead>
<tr>
<th>Phytoplankton species</th>
<th>Rainy Period</th>
<th>Dry Period</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Site 1</td>
<td>Site 2</td>
</tr>
<tr>
<td><strong>Bacillariophyceae</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Aulacoseira granulata</em> (Ehrenberg) Simonsen</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td><em>Aulacoseira distans</em> (Ehrenberg) Simonsen</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td><em>Cyclotella meneghiniana</em> (Kutzing)</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td><em>Cyclotella stelligera</em> Cleve (Cleve e Grunov)</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td><em>Frustulia rhomboides</em> (Ehremberg) De Toni</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td><em>Navicula bacillum</em> Ehrenberg</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td><em>Navicula crytocephata</em> Kutz</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td><em>Navicula viridula</em> (Kutz) Ehrenberg</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td><em>Nitzschia linearis</em> Smith</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td><em>Surirella capronii</em> Brébisson</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td><em>Synedra</em> sp</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td><strong>Chlorophyceae</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Ankistrodesmus fusiformis</em> Corda</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td><em>Botryococcus Braunii</em> Kutzing</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td><em>Botryococcus protuberans</em> West and West</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td><em>Chlorella vulgaris</em> Beijerinck</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td><em>Closterium parvulum</em> Näg.</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td><em>Coelastrum</em> sp</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td><em>Dictyosphaerium pulchellum</em> Naegli</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td><em>Oocystis borgei</em> Snow</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td><em>Oocystis lacustris</em> Chodat</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td><em>Pandorina morum</em> Bory</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td><em>Pediastrum duplex</em> Meyen</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td><em>Radiococcus</em> sp Midle</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td></td>
<td>Rainy Period</td>
<td>Dry Period</td>
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<td>--------------------------</td>
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<td>------------</td>
</tr>
<tr>
<td></td>
<td>Site 1</td>
<td>Site 2</td>
</tr>
<tr>
<td><em>Scenedesmus acunts</em> Meyen</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td><em>Scenedesmus dimorphus</em> (Turp.) Kutz</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td><em>Scenedesmus quadricauda</em> (Tupin) Brébisson</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td><em>Staurastrum leptocladium</em> Nordst.</td>
<td></td>
<td>x</td>
</tr>
<tr>
<td><em>Staurodesmus triangulares</em> (Smith) Tham</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td><strong>Cyanophyceae</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Anabaena circinalis</em> (kütz) Ralb</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td><em>Anabaena planktonica</em> Lemm</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td><em>Cylindrospermopsis raciborskii</em> Horecká</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td><em>Chroococcocus turgidus</em> (Kutz.) Nag</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td><em>Microcystis</em> sp</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td><em>Oscillatoria articulata</em> Gardner</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td><em>Oscillatoria lacustris</em> (Kleber.) Geitler</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td><em>Oscillatoria limnetica</em> Lemmerman</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td><em>Pseudoanabaena catenata</em> Laut.</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td><strong>Dinophyceae</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Gonyaulax</em> sp</td>
<td></td>
<td>x</td>
</tr>
<tr>
<td><em>Gymnodinium</em> sp</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td><em>Peridinium volzii</em> Lemmermann</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td><strong>Euglenophyceae</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Euglena acus</em> Ehrenberg</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td><em>Phacus tortus</em> (Lemmermann) Skvortzov</td>
<td>x</td>
<td>x</td>
</tr>
</tbody>
</table>

Figure 5. Ecological indices of phytoplankton during the rainy and dry seasons.
Table 3. Significant F-values (p <0.05) from one-way Anova and Kruskal-Wallis test for spatio-temporal variations of physical-chemical characteristics and chlorophyll *a* concentrations in the Reservoir Ministro João Alves, Brazil

<table>
<thead>
<tr>
<th></th>
<th>Rainy period</th>
<th>Dry period</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DF*</td>
<td>SS*</td>
</tr>
<tr>
<td>pH</td>
<td>1</td>
<td>0.0015</td>
</tr>
<tr>
<td>Temperature</td>
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<td>0.72</td>
</tr>
<tr>
<td>Dissolved oxygen</td>
<td>1</td>
<td>0.50000</td>
</tr>
<tr>
<td>Electrical cond.</td>
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<td>41905.1</td>
</tr>
<tr>
<td>Transparency</td>
<td>1</td>
<td>0.0002</td>
</tr>
<tr>
<td>Nitrate</td>
<td>1</td>
<td>0.00905</td>
</tr>
<tr>
<td>Orthophosphate</td>
<td>1</td>
<td>0.00109</td>
</tr>
<tr>
<td>Ammonia</td>
<td>1</td>
<td>*1.33</td>
</tr>
<tr>
<td>Chlorophyll <em>a</em></td>
<td>1</td>
<td>1081.13</td>
</tr>
</tbody>
</table>

*Kruskal-Wallis.

GL: degrees of freedom, SS: statistically significant, MS: median significant.
Table 4. Significant t-test values (p<0.05) for spatio-temporal variations of physical-chemical characteristics and chlorophyll $a$ concentrations

<table>
<thead>
<tr>
<th></th>
<th>Rainy period</th>
<th></th>
<th>Dry period</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean P1 (±Std)</td>
<td>Mean P2 (±DP)</td>
<td>t-value</td>
<td>p-value</td>
</tr>
<tr>
<td>pH</td>
<td>8.27 (±0.62)</td>
<td>8.24 (±0.75)</td>
<td>0.06</td>
<td>0.957</td>
</tr>
<tr>
<td>Temp.</td>
<td>30 (±1.51)</td>
<td>29.4 (±1.45)</td>
<td>0.57</td>
<td>0.589</td>
</tr>
<tr>
<td>D.O</td>
<td>8.55 (±0.50)</td>
<td>8.05 (±0.89)</td>
<td>0.97</td>
<td>0.369</td>
</tr>
<tr>
<td>Elec. Cond.</td>
<td>743 (± 44.1)</td>
<td>637.75 (±21.5)</td>
<td>0.43</td>
<td>0.684</td>
</tr>
<tr>
<td>Transp.</td>
<td>0.59 (±0.17)</td>
<td>0.60 (±0.18)</td>
<td>-0.08</td>
<td>0.940</td>
</tr>
<tr>
<td>Nitrate</td>
<td>0.35 (±0.18)</td>
<td>0.42 (±0.18)</td>
<td>-0.53</td>
<td>0.617</td>
</tr>
<tr>
<td>Orthophosphate</td>
<td>0.56 (±0.12)</td>
<td>0.54 (±0.12)</td>
<td>0.27</td>
<td>0.798</td>
</tr>
<tr>
<td>Ammonium</td>
<td>0.11 (±0.006)</td>
<td>0.08 (±0.04)</td>
<td>0.93</td>
<td>0.388</td>
</tr>
<tr>
<td>Chloro $a$</td>
<td>28.25 (±4.85)</td>
<td>5 (±3.55)</td>
<td>7.72</td>
<td>0.0002</td>
</tr>
</tbody>
</table>
Table 3 shows the results of one-way ANOVA, which is singularly significant in relation to chlorophyll $a$ levels between sites 1 and 2. Significant t-test values ($p<0.05$) for spatio-temporal variations of physical-chemical characteristics and chlorophyll $a$ concentrations are given in Table 4.

**DISCUSSION**

The present paper documents the impact of Nile tilapia cage culture on the successional pattern of phytoplankton species composition on spatial scale in the shallow tropical reservoir of Northeastern Brazil. This finding of potential effect of tilapia cage culture highlights the discussion on three broad contexts: Firstly, the inorganic nutrient levels in site 1, where the point source nutrient is significant when compared to the site 2 of the non-point nutrient source. Secondly, the effect of the low N:P ratio on the dominant cyanobacterial species is robust and persistent. Finally, the influence of flushing of reservoir during rainy season and the subsequent shift in phytoplankton species composition on temporal scale is significant.

The relative dominance of phytoplankton varied exceptionally from cyanobacterial species dominance in the littoral region (site 1) to diatoms dominated pelagic zone (site 2), which was a different situation from the one observed in an earlier study, where the dominance of diatom population was overwhelming in all zones (Nascimento, 2003; Chellappa et al., 2006). However, the present results are similar to the observation of Borges et al. (2010), where the dominance of cyanobacteria emanated from fish cage-culture practice.

International experience on Lake Aquaculture indicates that the cage culture is well established in Chile, despite environmental problems due to nutrient enrichment and organic waste impacts (EAO, 1997).

In southern Australia, particularly in Tasmanian lakes, diverse trophic status had been reported. The effects of cage culture resulted either in oligotrophic status with relatively low algal biomass or remained as mesotrophic (e.g. Lakes Sorell and Crescent), well mixed and with abundant phytoplankton.

Few cases have been reported as eutrophic at some stage (e.g. Craigbourne Dam). The eutrophic lakes have been associated with algal blooms (both blue-green and green) for varying periods, which are highly susceptible to changes in nutrient status, chlorophyll $a$ levels and TP concentrations (Davies, 2000; Sanger, 1992). The present paper when compared to these studies, indicates that low N:P ratio at point source site exhibited selective eutrophication during dry period, and consequently non-toxic cyanobacterial species appeared without resulting in fish mortality.

Fish kills have been registered in other reservoirs of Northeastern Brazil (Chellappa et al., 2000; Chellappa and Costa, 2003; Chellappa and Chellappa, 2011). The situation in the non-point source of the pelagic region was totally opposite, where species of diatoms and chlorophyceae dominated and the environment was free from the impact of cage culture effluents.

Another ecological explanation offered is the regime shift of temporal changes during the time when influx of river flow to reservoir increases considerably and stimulates phytoplankton species richness. This explanation considers that abrupt and persistent shift could be induced through eutrophication related drivers (Jeppesen et al., 1998; Crossetti and Bicudo, 2008).
The shift may be from prokaryotic filamentous cyanobacterial dominance to unicellular and colonial eukaryotic phytoplankton species (Scheffer et al., 1997). However, in the present study such changes were observed as an impact from cage-culture effluent, which is selective and significant only at point source site with the persistent dominance of *Anabaena circinalis* and *A. planktonica*. The reduction of species richness is partially in agreement with the results from semiarid Mediterranean phytoplankton species composition with cyanobacterial dominance (Barone et al., 2010).

Phytoplankton biomass is represented by the chlorophyll $a$ levels and is always linked to the light limitation factor in northern hemisphere reservoirs. Whereas, in semiarid tropical climate, light is year around abundant and dissolved inorganic nutrients levels of nitrogen and phosphorus act as strong environmental drivers in increasing chlorophyll $a$ concentrations.

The present study clearly indicates that increased nutrients especially orthophosphate in the vicinity of fish cages enhanced levels of chlorophyll $a$ in point source site than in non-point source site of the pelagic region.

As a response to two opposing resource-gradients, a situation similar to the high values registered in Scottish Lochs (SEPA, 1997) was observed in this study. Significant resource-gradient differentiation in spatial as well as temporal distribution of chlorophyll $a$ was registered in the point source site and non-point source (P < 0.05).

This study deals with comparative analysis of phytoplankton species composition based on spatial and temporal scale for an annual cycle in a tropical reservoir, wherein Nile tilapia cage culture was practiced by local fishermen. The effluents which originated from the cage culture of tilapia showed increased levels of nitrogen and phosphorus with more of low N:P ratio. Site 1 presents predominant growth of filamentous cyanobacterial species with no harmful effects to fish but resulting in reduced phytoplankton diversity. The site 2 revealed the dominance of diatoms, presence of some fast growing species of chlorophyceae and improved species diversity.

The levels of chlorophyll $a$ are higher in the site 1 than in site 2. These spatial variations in structural and functional components of phytoplankton and are attributed to opposing resource gradients of site 1 and site 2. This is an important social inclusion project sustained by the local municipality to promote sustained development project of cage-culture to improve the standards of fishermen community of the region.

The report from the present study might be considered as useful proposal of a best practice environmental management approach to reservoir fish farming through moderate cage culture, with an emphasis on methods to reduce potential risks (toxic cyanobacterial) and impacts (related to eutrophication).

**ACKNOWLEDGMENTS**

This study was supported by the National Council for Scientific and Technological Development of Brazil (CNPq) in the form of Research grants (N.T. Chellappa, S. Chellappa and R. V. Santos) and by the Foundation CAPES/MEC (scholarship awarded to E. K. R. Pessoa during the study period).
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OVARIAN DEVELOPMENT AND REPRODUCTIVE PERIOD OF WHITE MULLET, *Mugil curema* IN THE COASTAL WATERS OF NORTHEASTERN BRAZIL

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**ABSTRACT**

White mullet, *Mugil curema* is a coastal pelagic fish and is an important fishery resource of the Atlantic Ocean and is a component of artisanal fisheries in northeastern Brazil. This study reports on the ovarian development and reproductive period of *M. curema* (Osteichthyes: Mugilidae) using both macroscopic and histological techniques. The length-weight relationship, size at sexual maturity and gonadosomatic index (GSI) of females were determined. The females show isometric growth and the size at first sexual maturity was at 24.3 cm of total length. The macroscopic characteristics of the ovaries revealed four stages of development: immature, in maturation, mature and spent and the microscopic features showed five stages: immature, initial maturation, final maturation, mature and spent. *M. curema* has a prolonged reproductive period, with two peaks of reproductive activity in January-February and in May, coinciding with increased rainfall.

**Keywords:** ovarian development, reproductive period, size at first sexual maturity, oocyte development

**INTRODUCTION**

Mullets belonging to the family Mugilidae are distributed in coastal waters, estuaries and freshwater environments in tropical and subtropical regions throughout the world (Harrison, 2003; Nelson, 2006). They are considered as important economic resources that support many small communities through both fishing and aquaculture (Pina and Chaves, 2005; Katselis et al., 2005).

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Among the seven species of mullets which occur in Brazil, *Mugil curema*, *M. liza* and *M. platanus* are commercially exploited (Menezes, 1983). White mullet, *M. curema* is a coastal pelagic fish which occurs in the Western Atlantic (Nova Scotia to Argentina), Eastern Atlantic (Senegal to Namibia), and in the Eastern Pacific (California to Chile) (Harrison, 2002). It is an important component of artisanal fishery and is a source of income and food for people depending on fishing in the coastal region of Brazil.

Conservation of fish stocks in their natural habitat are usually endangered by abusive fishing of immature fishes which have not yet completed their reproductive cycle, as recruitment via reproduction is the means by which the resource is renewed. The Fishery Research Program of Northeastern Brazil (REVIZEE-SCORE-NE) has carried out various fishery surveys to determine the conservation status of the fish stocks in their natural habitats (Lessa, 2006). Fishery surveys carried out in the northeastern continental shelf waters emphasize the importance of the flying fish, *Hirundichthys affinis*, thread herring, *Opisthonema oglinium* and *M. curema* as important fish resource for the State of Rio Grande do Norte, Brazil (IBAMA, 2007).

Reproductive biology of white mullet, *M. curema* was investigated in Margarita Island, Venezuela (Marín et al., 2003); in a coastal lagoon in the Gulf of Mexico (Ibáñez-Aguirre and Gallardo-Cabello, 2004), in the southern Caribbean (Solomon and Ramnarine, 2007). Gonadal development and spawning season of *M. curema* was investigated in southeastern Brazil (Albieri et al., 2009; Albieri et al., 2010). Yet for all, there exists a lacuna of information dealing with ovarian development and reproductive period of *M. curema* in the Northeastern coastal waters of Brazil.

The present investigation aimed to (i) examine the length-weight relationship of female *M. curema*; (ii) determine length at first maturity of females; (iii) estimate fecundity, ovarian developmental stages, type of spawning, relate maturity stages to gonadosomatic index and correlate the reproductive period with rainfall. This information could contribute for the management of this important fishery resource in the coastal waters of Northeastern Brazil.

**MATERIALS AND METHODS**

**Study Site and Sample Collection**

Specimens of *M. curema* were collected monthly during the period August 2008 to July 2009, off Rio Grande do Norte, in the Atlantic coastal waters of Brazil (Figure 1). The specimens were captured using beach seine nets, which were 110 m long, 3 meters in height with a central mesh of 10 mm and 70 mm at the extremities. The beach seine was taken 100 m from the beach by local fishermen and released at a depth of 5 m with the aid of a non-powered local craft.

After the capture, each specimen was measured (total length in cm TL) and weighed (total body mass TW in g). The fish were dissected and the gonads were removed for identification of sex and to assess the stage of development. A total of 180 females collected were separated for detailed studies. Rainfall data of the region was obtained from Agricultural Research Company of Rio Grande do Norte (EMPARN), Brazil.
Length – Weight Relationship of Females

The length-weight relationship was determined by the equation: \( W = a L^b \), where \( W \) is the total weight (g), \( L \) is the total length (cm), \( a \) is the interceptor (initial growth coefficient or condition factor) and \( b \) is the slope (growth coefficient or fish relative growth rate) (Hayes et al., 1995; Jobling, 2002; Santos et al., 2002). The parameters \( a \) and \( b \) of \( L - W \) relationships were estimated using a power function in which was used the coefficient of determination \( r^2 \) to measure the degree of association between two variables (\( L \) and \( W \)).

Length at First Maturity (\( L_{50} \)) of Females

To determine the size at first sexual maturity, the gonads of mature females were pooled (maturing, mature and spawned), followed by distribution of the accumulated relative frequencies by total length classes (Moreno et al., 2005).
Macroscopic Description of Ovaries and Histology and Oocyte Development

The characteristics used for macroscopic classification of the ovaries were according to Vazzoler (1996) and Murua et al. (2003). The following external aspects were considered: size, form, colour, presence of blood vessels, oocytes visible to naked eye, rigidity, turgidity and the space occupied in the body cavity.

In order to avoid possible variation in the developmental stage of oocytes due to their position in the ovaries, histological examinations were carried out on sections from the anterior (cephalic), middle (central), and posterior (caudal) regions of 20 ovaries in different developmental stages (Yoshida, 1964). Fragments of ovaries selected for histological analysis were fixed in Bouin solution for 12-24 h (depending on size), washed for 24 hours in running water to remove excess fixative and were later preserved in 70% alcohol. Fragments of ovaries selected for histological study were embedded in paraffin, sectioned at 3-5 μm thickness, and stained with Hematoxylin-Eosin (HE). The histological description of the stages of oocyte development and classification of microscopic stages of ovarian maturation was performed using the terminology described by West (1990) and Vazzoler (1996).

Fecundity and Type of Spawning

The oocytes from mature ovaries were dissociated using Gilson solution and three sub-samples of 0.1 g were extracted and the oocytes were counted using Bogorov plates, a stereomicroscope and an ocular micrometer. Total fecundity was estimated for the total weight of the ovaries. Fecundity = [(number of mature oocytes in the fragments of ovary) x (total weight of ovary)] / (weight of the fragment of the ovary). Absolute fecundity was estimated using mature ovaries weighing on an average 37.96 g of females with average total length of 30.6 cm. The type of spawning was assessed by measuring the oocyte diameter (in μm) and by histological analysis of the oocyte development (Vazzoler, 1996).

Gonadosomatic Index and Reproductive Period

The gonadosomatic index (GSI) of females was calculated using the equation: GSI = (Wg / Wt) * 100, where Wg is the gonad weight (g), and Wt is the total weight of the individual (Wootton et al., 1978). The reproductive period was determined based on the distribution of relative frequencies (%) of each maturity stage of gonads and the variation of average monthly gonadosomatic index (GSI).

RESULTS

Sample Size

A total of 366 samples of *M. curema* were captured during the study period, of which 50.8% (n = 186) were males and 49.1% (n = 180) were females. Only the females were used in this study.
The frequency distribution of occurrence by total length classes indicate that females ranged from 15.6 to 34.5 cm (24.9 ± 4.1) (Figure 2a). About 35% of females sampled were in the length classes of 19-21 and 21-23 cm and were considered as young females. In terms of body mass, females ranged from 35.4 to 382g (160.5 ± 82.3g) (Figure 2b).

Figure 2. Frequency of occurrence of female *M. curema* by total length classes (a) and body mass (b).
Length-Weight Relationship of Females

The resulting function of L-W relationships for females were $W = 0.0099L^{2.985}$, ($r^2 = 0.97$) (Figure 3), with a positive correlation between total length and body mass, thus presenting a coefficient of 2.985 indicating isometric growth.

![Figure 3. Length-Weight relationship of females of *M. curema*.

Length at first maturity (L_{50}) of Females

The analysis of cumulative frequency distribution by total length for the adult samples of *M. curema* females revealed that the minimum length at first sexual maturity (L_{50}) was 24.3 cm of total length (Figure 4).

![Figure 4. Length at first sexual maturity of female *M. curema*.](image)
**Macroscopic description of ovaries**

![image of ovaries](image)

Figure 5. Macroscopic stages of ovarian development of *M. curema* (a) immature, (b) maturing, (c) mature, (d) spent. (Scale: 1 cm).

The ovaries of *M. curema* presented as paired structures, elongated and lobulated with weights ranging from 0.004 g to 48.5 g (3.0 ± SD 8.34 g). The shape, size, weight, blood vessels and staining varied during the ovarian development (Table 1). In the macroscopic observations of the ovaries the following four developmental stages were observed: immature, maturing, mature and spent (Table 1) (Figure 5).

**Histology and Oocyte Development**

Five stages of ovarian development were determined microscopically for *M. curema* according to each growth stages of oocytes (Table 1) (Figure 6). The development of oocytes was classified into two phases.

The first phase of development or pre-vitellogenic phase includes the young germ cell (stage I) and perinucleolar (stage II). The second phase of development or the vitellogenic phase, includes lipid vitellogenesis (stage III), vitellogenesis of lipid and protein (stage IV) and post-vitellogenesis (stage V).

Young germ cell or chromatin nucleolus stage (Stage I). The smallest cells were found (<10 µm), which appear grouped in nests placed in the ovigerous lamellae, with little cytoplasm, usually with a single central rounded nucleolus, intensely basophilic. These cells were observed in immature ovaries (Figure 6 a).

Early perinucleolus stage or reserve stock (Stage II). The nucleus was in the centre, initially with one or two spherical nucleoli (intensely basophilic) and the cytoplasm was well defined and more basophilic than in the previous phase, measured 10-50 µm.
Late perinucleolus stage (Stage II). The nucleoli become more numerous and were present in the periphery of the nucleus. The cytoplasm is less basophilic showing oocytes of all stages of maturity, measuring 40-70 µm (Figure 6b).

Lipid vitellogenesis (Stage III). The oocytes measured 70-150 µm, showing the central nucleus, cytoplasm is less basophilic than in the previous phase, and with a vacuole representing the lipid deposition. Found mainly in early maturing ovaries (Figure 6b).

Lipid and protein vitellogenesis (Stage IV). The oocytes measured 170-250µm, showing the deposition of protein in the form of platelets from peripheral acidophilic cytoplasm. This stage was present in final maturation of the ovaries (Figure 6c). Post-vitellogenesis (Stage V). The oocyte rapidly increases in size, depending on the accelerated increase in the number of acidophilic yolk granules. The diameter of the oocytes was from 320 to 510µm. This stage was present in mature ovaries (Figure 6d).

Fecundity and Type of Spawning

Absolute fecundity estimated for mature ovaries was 245,828 oocytes. Relative fecundity was estimated at 814 oocytes per gram body mass of the fish. A high frequency of
oocytes with complete vitellogenesis along with perinucleolar oocytes, were observed in histological sections of mature ovaries of *M. curema*. The presence of two batches of oocytes was observed in mature ovaries: the reserve stock of ovogonia and mature oocytes, which indicate the synchronous oocyte development in two groups, characteristic of total spawners.

**Table 1. Macroscopic and histological classification and description of the ovarian maturity stages of female *M. curema***

<table>
<thead>
<tr>
<th>Maturity stage</th>
<th>Macroscopic description</th>
<th>Microscopic description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immature</td>
<td>Ovaries are small, translucent and oocytes were not visible to the naked eye (Fig. 5a).</td>
<td>Nest of germ cells (oogonia) compactly fill the ovaries and clusters of very small oocytes are beneath the ovigerous lamella (Fig. 6a).</td>
</tr>
<tr>
<td>Maturing</td>
<td>The ovaries are larger, lobulated in shape, with a slight reddish tinge and occupying about 1/3 to 2/3 of the coelomic cavity. Blood vessels and small opaque oocytes were visible to the naked eye (Fig. 5b).</td>
<td>Early stage: occurrence of early perinucleolus and late perinucleolus oocytes. Initially the nucleus is with a nucleolus and later appears numerous nucleoli. Oocytes are in previtellogenic stage (Fig. 6b). Late stage: oocytes are mostly with yolk granules and oil vesicles. Reserve stock of germ cells and oocytes with lipid and protein granules are present (protein granules are pink in colour) (Fig. 6c).</td>
</tr>
<tr>
<td>Mature</td>
<td>Ovaries are turgid in appearance, orange in colour and occupy 1/2 of the coelomic cavity. Numerous larger opaque oocytes with intense superficial vascularization. (Fig. 5c).</td>
<td>Mature oocytes are predominant, with yolk plates. Reserve stock of germ cells separated in nests are present (Fig. 6d).</td>
</tr>
<tr>
<td>Spent</td>
<td>Ovaries are elongated, flaccid in appearance and reddish-brown in colour (Fig. 5c).</td>
<td>Ovaries show hemorrhaging areas and empty spaces. Reserve stock of germ cells and residual oocytes in the reabsorbing process are present (Fig. 6e).</td>
</tr>
</tbody>
</table>

**Frequency of Occurrence of Ovarian Maturity Stages**

The frequency of occurrence of ovarian developmental stages showed that females in the immature and maturing stages occurred throughout the year. Mature females were observed from October, 2008 to July 2009, excepting the month of December. Spawned individuals were
registered during the months of October, 2008 to July 2009, excepting the months of November and March (Figure 7).

![Bar chart showing monthly frequency of ovarian maturity stages in M. curema captured during August 2008 to July 2009.](image)

**Figure 7. Monthly frequency of ovarian maturity stages in M. curema captured during the period August, 2008 to July, 2009.**

### GSI, Reproductive period and rainfall

The GSI of females ranged from 0.20 to 4.23 (1.23 ± 1.21). During the study period rainfall varied from a minimum of 0.4 mm in December, 2008 to a maximum of 427.9 mm in August, 2008 (208.2 ± 154.8). Northeastern Brazil experienced 4 months of drought from September to December (19.9 ± 17.7), and 8 months of rain from January to August (302.3 ± 84.6).

The highest values of GSI of females were registered during the rainy season and lowest during the dry season (ρ =0.35; p = 0.243) (Figure 8). The presence of mature individuals during October to July and increased values of GSI, specifically in January, February and May 2009, shows that females have a prolonged period of reproductive activity, with two clear peaks, the first in January-February and the second in May, coinciding with increased rainfall.

### DISCUSSION

Females of *M. curema* in smaller classes of total length were abundant in the coastal waters where they were captured. This possibly suggests that this ecosystem offers adequate conditions for the development and survival of the juveniles. Quiñonez-Velázquez and López-Olmos (2011)
observed that early juveniles of white mullet have greater growth rates in the coastal area, but with greater risks of mortality. The growth of juveniles is more rapid in the coastal zone than in the lagoon, and the strategy of moving to protected areas is to reach a length that maximizes escape from predation when they return to the coastal areas where the conditions for growth are better and probability of survival is maximized.

Growth is an important factor for establishing optimal fishery management and particularly for understanding the population dynamics of species (Ferrer Montaño and Villasmil, 2008). It is possible to determine the type of growth of a species using the allometric coefficient ($b$) (Jobling, 2002). The L-W relationships of *M. curema* indicate isometric growth within the expected range of 2.5-3.5. The parameters of L-W relationships in fish are affected by factors such as environmental conditions, gonad maturity, sex, stomach fullness, health condition, season, population and differences within species (Froese, 2006).

For the rational management of a fishery stock subject to exploitation, it is of basic importance to know the size at first gonadal maturation ($L_{50}$), since it provides information for determining the minimum size at capture and mesh dimensions. The $L_{50}$ value of 24.3 cm obtained in this study is in accordance with that recorded by Marín et al. (2003) for the same species in Venezuela. However, $L_{50}$ value of 27.8 cm for females of the same species was registered by Ibáñez-Aguirre and Gallardo-Cabello (2004). The size at first maturity is not fixed and may vary between individuals of the same species, whose populations are subject to different environmental conditions (Wootton, 1990). The size at first maturity can be anticipated with a better food supply, since this leads to an increase in growth rate (Nikolsky, 1963).

The macroscopic anatomy of the ovaries of *M. curema* conforms to the descriptions of Albieri et al. (2009) for the same species. Solomon and Ramnarine (2007) observed for *M. curema* six macroscopic stages of ovarian development: resting, early maturing, late maturing, mature, partially spawned and spawned. The use of such macroscopic scales contribute to the biological knowledge, but the use of histological analysis and measurement of oocytes reduce the risk of incurring errors (West, 1990).

The five stages of oocyte development and the processes of ovarian development of *M. curema* were verified in this study. Oocytes in the pre-vitellogenic stage were present throughout the reproductive cycle in the ovaries, which are regarded as reserve stock for the subsequent breeding season. When there are two groups of oocytes in the ovaries in maturation, development is considered as synchronous (Wallace and Selma, 1981; Murua and Saborido-Rey, 2003). The present study confirms that *M. curema* has synchronous oocyte development in two groups. However, Somolon and Ramnarine (2007) observed that ovaries of individuals before spawning showed primary oocytes, oocytes with yolk formation and vitellogenic oocytes indicating synchronous development in the three groups.

The value of fecundity estimated for *M. curema* in this study approximates with that of other studies (Ibáñez-Aguirre and Gallardo-Cabello, 2004). A study conducted in the Caribbean for *M. curema* indicates that it is a partial spawner and females spawned twice within a reproductive period (Solomon and Ramnarine, 2007). Present study registers total spawning for *M. curema* in the northeastern coastal waters of Brazil, in agreement with the results of Albieri et al. (2009).

The highest values of GSI and greater occurrence of mature females indicated the reproductive period. The GSI is a very effective indicator to determine the degree of development and reproductive period of fish (Isaac-Nahum and Vazzoler, 1987;
This study registered a prolonged reproductive period for *M. curema*, with peaks coinciding with increased rainfall. In general, inshore species in lower latitudes exhibit a longer spawning period (Longhurst and Pauly, 1987). Marin et al. (2003) recorded mature individuals of *M. curema* throughout the year in the island of Margarita, Venezuela and successful spawnings coincided with the end of the rainy season. In tropical environments, temperature and photoperiod have low seasonal variations, however seasonal changes of wind and rain cause some seasonality (Lowe-McConnell, 1987). In tropical regions the rainfall plays an important role in determining the reproductive cycles of fish and collective reproduction occurs during the time when environmental conditions are favourable for the survival of juvenile forms and when adequate food is available, besides protection from predators (Chellappa et al., 2010). *M. curema* has a prolonged reproductive period, with two peaks of reproductive activity in January-February and in May, coinciding with increased rainfall.

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The authors wish to thank the Federal Post-Graduate Agency of Brazil (CAPES/MEC) (Scholarship granted to M.R. Oliveira) and the National Council for Scientific and Technological Development of Brazil (CNPq) for the Research grants awarded during the study period (E.F.S. Costa and S. Chellappa).

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ANTIOXIDANT AND DETOXIFICATION ACTIVITIES OF GILL AND DIGESTIVE GLAND OF LAMELLIDENS MARGINALIS (MOLLUSCA: BIVALVIA) EXPOSED TO AZADIRACHTIN

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ABSTRACT

*L. marginalis* is an important biological component of freshwater ecosystem and an efficient bioaccumulator of toxin residues. Being a filter feeder, this species performs biological filtration of ambient water and keeps the water body biologically safe for other species.

Natural habitat of the species is under risk of contamination by multineem, a newly introduced azadirachtin (limonoid) based pesticide. During monsoon, natural habitat of *L. marginalis* received residues of diverse xenobiotics along with azadirachtin. Reactive oxygen intermediates are major cytotoxic factor which is generated in invertebrates in diverse physiological conditions.

The stimulation of superoxide anion production and resulting physiological damage is modulated by the presence of antioxidant enzyme namely superoxide dismutase (SOD) and catalase (CAT).

Further the toxin residues are conjugated in glutathione pool through glutathione-S-transferase (GST). This is a protective phenomenon in bivalves. In *L. marginalis*, the activity levels of antioxidant enzymes namely superoxide dismutase and catalase activities were significantly low than control in gill and digestive gland under toxin exposure respectively. In case of glutathione-S-transferase, azadirachtin moderately suppress the activity of the enzyme in selected tissues of *L. marginalis*.

Keywords: Bivalve, Azadirachtin, Antioxidant and detoxification enzymes

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INTRODUCTION

Molluscs are of interest not only to the farmers and horticulturists but to the ecotoxicologist as biomonitor species for environmental pollution (Ebenso, 2004; Chakraborty et al., 2010). They encounter toxic materials either by contact through medium or during feeding. In recent years there has been a considerable interest in the use of biochemical indicators within bivalve mollusc in contrast to previous monitoring studies, which mainly focus on the level of contaminants. *L. marginalis* has been identified as an efficient filter feeder bivalve (Das and Jana, 1999) and is widely distributed along the floors of the freshwater bodies. Azadirachtin is a natural bioactive agent derived from the seeds of the neem tree *Azadirachtin indica* (Schmutterer, 1990). Azadirachtin is a triterpenoid compound and expresses insecticidal activity against a broad range of insect pests (Singh and Singh, 1995). The insecticidal activity involves pest repellence, feeding and ovipositional deterrence and insect growth regulation (Schmutterer, 1990). ‘Multineem’ is an azadirachtin based biopesticide which is being recently used by the Indian farmers for the purpose of protection of crops from insect pest attack. Indiscriminate application of insecticide to agricultural field may result in contamination of adjacent freshwater bodies leading to exposure of nontarget organisms like *L. marginalis* to toxic azadirachtin (Isman, 1997).

An important body of investigation has focused on biomarkers of oxidative stress within organisms. Antioxidants enzyme systems (superoxide dismutase (SOD), catalase (CAT)) and detoxification enzyme like glutathione-S- transferase (GST) are efficient protective mechanisms against chemical reactive species produced by endogenous metabolism or by the biotransformation of xenobiotics. The reactive oxygen species (ROS) are generated in haemocytes and tissues in molluscs as superoxide anion (O$_2^-$) having microbicidal activity and are rapidly dismutated by the action of metalloenzyme superoxide dismutase to hydrogen peroxide and oxygen. Hydrogen peroxide is continuously generated by several enzymes including SOD which must be degraded to prevent oxidative damage (Gamble et al., 1995; Isani et al., 2000). The antioxidant catalase, is a hematin-containing enzyme based in the peroxisomes of cells and is an important component of intracellular and antioxidant defences of aquatic organisms (Jamil, 2002). It reduces the hydrogen peroxide into water and oxygen to prevent oxidative stress and in maintaining cell homeostasis (Arun and Subramanian, 1998; Arun, 2000). The decomposition of hydrogen peroxide is directly proportional to both the concentration of enzyme and the concentration of substrate (Di Giulio et al., 1989). Peroxide, including hydrogen peroxide is one of the main reactive oxygen species (ROS) leading to oxidative stress (Gamble et al., 1995). The cytotoxic effect of H$_2$O$_2$ is thought to be caused by hydroxyl radicals generated from iron catalyzed reactions causing subsequent damage to DNA, proteins and membrane lipids. In molluscs, catalase catalyzes the conversion of H$_2$O$_2$ to H$_2$O and O$_2$ (Scott et al., 1991; Arun, 2000). Antioxidant and detoxification responses have evolved as adaptive response in animal series. GST is an effective enzyme involved in conjugation phase of detoxification response. Glutathione-S transferase catalyzes the conjugation of reduced glutathione to nucleophiolic xenobiotics (Ishizuka et al., 1998; Nielsen et al., 1999). GST primarily is involved in the chemical disposition of toxic substances and has the ability to catalyse the conjugation of GSH to various toxic electrophiles and inactivate toxic compounds by non catalytic binding (Lamoureux and Rusness, 1987) and elimination from the system. The present study evaluates the influence of
a biopesticide on the antioxidant and detoxification enzyme activities in the gill and digestive gland of the freshwater bivalve, *L. marginalis*.

**MATERIAL AND METHOD**

**Collection and Treatment of Animals**

The adult healthy *L. marginalis* with shell size of 7-8 cm were manually collected from the selected wetlands of the district of South 24 Parganas of West Bengal. Animals were transported to the laboratory in rectangular plastic containers with a dimension of 12’x18’x 6’ at a density of 4-6 individuals per box in moist condition. Prior to experimentation, animals were acclimatized for 15 days in the laboratory.

During acclimatization, *L. marginalis* were maintained in aquaria with fresh supply of pond water with temperature of 29°C±3°C and the animals received uniform ration of illumination. During the course of acclimatization and experiment, the animals were fed with chopped *Hydrilla* sp. and common aquatic weeds (Raut, 1991).

Routine replenishment of water was carried out in every 12 hours to avoid residual toxicity. Aqueous solutions of Multuneem (Multiplex, India Private Limited, Azadirachtin E.C. 0.03%) formulations were prepared in Borosilicate glass containers with azadirachtin concentrations of 0.006, 0.03, 0.06, and 0.09 ppm. The pH of the solution was maintained at 7.2. Each experimental set consisted of 10 animals of same shell length. Animals were exposed to a volume of 5 litre of pesticide solution for varied span of exposure i.e. 1,2,3,4,7,15 and 30 days. For control, a set of animals were kept in identical volume of pesticide free analytical grade water. The experiments were carried out in static water environment and fresh solutions of pesticide were replenished in every 12 hour.

**Preparation of Tissue Lysate**

Gills and digestive gland of *L. marginalis* was dissected in cold sterile saline and tissue of uniform weight (100 mg) was homogenized in 0.25 M sucrose solution (3%w/v) in mechanical glass homogenizer at 4°C and was centrifuged at 100x g for 15 minutes at cold (Vijayavel and Balasubramanian, 2006). Estimation of protein and enzyme were carried out from supernatant.

**Super oxide Dismutase (EC1.15.1.1)**

Activity of super oxide dismutase was determined in tissue lysate following the method of Krishnan *et al.* (2002). Assay involved reaction of gill and digestive gland suspension to Griess reagent followed by absorbance at 560 nm. One unit of enzyme activity is defined as the amount of SOD capable of inhibiting 50% of nitrite formation under assay condition. Activity of superoxide dismutase was calculated and expressed as unit of SOD/mg of protein/min.
Catalase (EC1.11.1.6.)

Catalase activity was estimated according to Prakash and Rao (1995). For test sample, the reaction mixture consists of 5 ml of hydrogen peroxide (6mM) as substrate and 0.5ml of enzyme (0.1% Triton-X lysed gill and digestive gland). For standard, the reaction mixture consists of 5.5ml of phosphate buffer (0.01M). For both test and standard sample, the reaction was stopped by adding 1ml of sulphuric acid (6N) followed by 7ml of potassium permanganate (0.01N) after 3 mins of reactions. The final reaction mixture was monitored at 480 nm for 30-60 secs after the stoppage of the reaction. The activity was expressed as µl of catalase/mg protein/min.

Glutathione -S Transferase (EC 2.5.1.13)

Glutathione-S transferase activity was estimated in the gill and digestive gland following the method of Aceto et al. (1991). One unit of GST refers to the enzyme amount needed to conjugate 1µM of 1 chloro 2, 4dinitrobenzene per min, per mg of total protein present in the homogenate. The enzyme activity was expressed as µM GST/ mg protein/ min.

RESULTS

Antioxidant Activity

The activity of SOD was highest in digestive gland followed by gill (Figure 1). The degree of inhibition of SOD in the tissues expressed a dose dependent response and considerable inhibition occurred even at low concentrations (0.006ppm) of azadirachtin exposure. Maximum inhibition in SOD activity was observed as 0.5 unit/mg of protein/min in the digestive gland as compared to control upon 0.09 ppm of azadirachtin exposure for 7 days (Figure 2).

Oxygen toxicity, referred to in earlier days as the oxygen toxicity is currently in focus in stress related environmental effect (Arun and Subramanian, 1998). The toxic effects of oxyradicals include lipid peroxidation, nucleic acid damage and enzyme inactivation. Self scavenging of these oxyradicals is an adaptive response which is studied by estimating catalase which is involved predominantly in the removal of hydrogen peroxide. Catalase has been used in aquatic organisms as a biomarker for monitoring environmental pollution and oxyradical induced damage.

In tissues of control animal, the activity of catalase was highest in the digestive gland followed by gill (Figure 4 and 3). The degree of inhibition of catalase in the tissues expressed a dose dependent response and considerable inhibition occurred at low concentrations of azadirachtin exposure (Figure 3 and 4).

Maximum inhibition in catalase activity was observed as 0.35 µm /mg protein/min in the digestive gland as compared to control for 0.09 ppm/7days exposure (Figure 4). In the gill, the maximum inhibition of the activity of catalase was recorded as 0.4µm /mg protein/min against 0.09 ppm of azadirachtin exposure for 7 days (Figure 3).
Figure 1. Activity of superoxide dismutase of gill of *L. marginalis* (7-8 cm shell length) exposed to azadirachtin *in vivo*. Data is represented as Mean±S.D. Statistical significance is shown at P<0.05*, P<0.01**, P<0.001***. (n=5).

Figure 2. Activity of superoxide dismutase of digestive gland of *L. marginalis* (7-8 cm shell length) exposed to azadirachtin *in vivo*. Data is represented as Mean±S.D. Statistical significance is shown at P<0.05*, P<0.01**, P<0.001***. (n=5).
Figure 3. Activity of catalase of gill of *L. marginalis* (7-8 cm shell length) exposed to azadirachtin *in vivo*. Data is represented as Mean±S.D. Statistical significance is shown at *P*<0.05*, *P*<0.01**, *P*<0.001***. (*n*=5).

Figure 4. Activity of catalase of digestive gland of *L. marginalis* (7-8 cm shell length) exposed to azadirachtin *in vivo*. Data is represented as Mean±S.D. Statistical significance is shown at *P*<0.05*, *P*<0.01**, *P*<0.001***. (*n*=5).

Detoxification Activity

Glutathione-S-transferase is involved in the detoxification reaction process of various xenobiotic chemicals. The activity level of GST was relatively lower than that of control throughout the study in all the tissues for azadirachtin exposure. In tissues of control animal, the activity of GST was highest in digestive gland followed by the activity in gill (Figure 5...
and 6). The degree of inhibition of GST in the tissues expressed a dose dependent response and considerable inhibition occurred at low concentrations of pesticide exposure (Figure 5 and 6). Azadirachtin of 0.09 ppm/7 days exposure resulted in maximum inhibition in GST activity which was determined as 1µm /mg protein/min in the digestive gland as compared to control (Figure 6). In the gill, the maximum inhibition of the activity of GST was recorded as 1.5 µm /mg protein/min against 0.09 ppm of azadirachtin exposure for 7 days (Figure 5).

Figure 5. Activity of GST of gill of *L. marginalis* (7-8 cm shell length) exposed to azadirachtin *in vivo*. Data is represented as Mean±S.D. Statistical significance is shown at P<0.05*, P<0.01**, P<0.001***. (n=5).

Figure 6. Activity of GST of digestive gland of *L. marginalis* (7-8 cm shell length) exposed to azadirachtin *in vivo*. Data is represented as Mean±S.D. Statistical significance is shown at P<0.05*, P<0.01**, P<0.001***. (n=5).
DISCUSSION

Molecular oxygen is an essential element of life and reactive oxygen species (ROS) are generated in all aerobes (Krishnan et al., 2002). Strategy of oxygen radical mediated killing is based on the premises of toxicity evolved due to high concentrations of molecular oxygen. Superoxide anions undergo reaction with hydrogen to produce hydrogen peroxide, hydroxyl radicals and finally water (Rodriguez and Moullac, 2000). Superoxide anions are extremely toxic, powerful and hyperactive killing agents which are capable of creating damage of the cells and tissues of self. Most ROS are generated as superoxide anion \((O_2^-)\) having microbicidal activity and are rapidly dismutated by the action of superoxide dismutase to hydrogen peroxide and oxygen. In this study, the activity of antioxidant enzyme namely superoxide dismutase is higher in digestive gland followed by in gills (Figure 2 and 1). Exposure to azadirachtin decreased the generation of superoxide dismutase for all four experimental concentrations in gill and digestive gland which expressed a dose dependent response. Since xenobiotic induced production of ROS vary significantly against environmental factors, necessary adjustments in antioxidant defences are required to maintain the steady state concentration of ROS for prevention and minimization of oxidative stress and cellular damage (Lesser, 2006) and is considered as a biochemical adaptive response. A decrement of superoxide dismutase production was observed against increasing azadirachtin concentrations. Catalase activity decreased significantly both in gill and digestive gland (Figure 3 and 4). In conclusion, present data demonstrate the sensitivity of antioxidant enzymes of \(L.\) marginalis under the exposure of azadirachtin. Azadirachtin induced decrement of antioxidant enzymes is indicative to a possible onset of physiological stress in the specimen distributed in water bodies contaminated with neem pesticide. Glutathione-S transferase which catalyzes the conjugation of reduced gluthathione with nucleophilic xenobiotics or cellular components damaged by oxyradical attack in their detoxification reaction (Ishizuka et al., 1998). Azadirachtin moderately suppressed the activity of the GST in haemocytes and selected tissues of \(L.\) marginalis (Figure 5 and 6) which is indicative of possible impairment of detoxification machinery. Toxin induced inhibition of GST activity would increase the magnitude of residual toxicity of azadirachtin accumulation in the selected tissues and organs of \(L.\) marginalis. Being a filter feeder, this species performs biological filtration of ambient water and maintain the water body biologically safe for other species. Molluscs, in recent years are gaining enormous interest among toxicologists as suitable biomarker of toxicity. This slow moving biofilter organism is capable of providing useful information of the health of waterbodies as suitable indicator of water pollution. Present investigation would help to establish a suitable biomonitor species of aquatic pollution with reference to toxicity of neem based pesticide and allied xenobiotics.

REFERENCES


