Effect of Heavy Metals on the Reproduction of Rasbora dandia

Thesis submitted to the University of Calicut in fulfilment of the requirement for the degree of Doctor of Philosophy in Zoology under the Faculty of Science

By

PRIYA RAJAN, M.Sc.

Under the supervision of

Dr. Balu T. Kuzhivelil

DEPARTMENT OF ZOOLOGY

CHRIST COLLEGE (AUTONOMOUS)

IRINJALAKUDA KERALA

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DECLARATION

I hereby declare that the thesis entitled **'Effects of heavy metals on the reproduction of *Rasbora dandia*'** is an authentic record of the original research work carried out by me under the supervision of Dr. Balu T. Kuzhivelil, Department of Zoology, Christ college, Irinjalakuda, and it has not been previously formed the basis for the award of any degree, diploma, associateship, fellowship or other title or recognition.

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CERTIFICATE

This is to certify that the thesis entitled "**Effects of Heavy Metals on the Reproduction of *Rasbora dandia***" is an authentic record of research work carried out by Ms **Priya Rajan** in the Department of Zoology, Christ College Irinjalakuda, under my guidance and supervision, in partial fulfilment for award of the degree of Doctor of Philosophy in Zoology of the University of Calicut.

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CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

Over the last a few decades pollution is increasing at tremendous rate. The aquatic ecosystems are the most affected from global pollution because final destination of pollutants released elsewhere on the earth is hydrosphere. Aquatic ecosystems are under the constant threat of pollutants released by various anthropogenic activities. Availability of water being a basic necessity of life, water pollution deteriorates the quality of life and wellbeing of human societies. Apart from the impact on human life, it is obvious that quality of water is crucial to various organisms in aquatic ecosystems. When aquatic biota becomes a food source, contaminants accumulated in the aquatic food chains influence the health of terrestrial organisms including the human beings.

Any chemical at a higher concentration can be an aquatic pollutant causing effects like discoloration, smell, temperature change, reduction in oxygen, altered pH, eutrophication and toxicity in water (Crathorne and Dobbs, 1990). Municipal sewage disposal and industrial discharge of effluents into rivers and sea are among the major point sources of water pollution. When waste waters from these sources are disposed into natural water bodies without sufficient treatments, physical and chemical disturbances occur in aquatic environment leading to ecosystem damage. Non point sources of aquatic pollution are more diffuse sources like agricultural run-off and atmospheric deposition. Agricultural practices have significant role in deteriorating the water quality of lakes, rivers and estuaries through nutrient contamination (Pucket, 1995; Moss, 2008, Parris, 2011).

Major contaminants of aquatic environment are comprised of both organic and inorganic chemical pollutants. Presence of pathogenic organisms as well as hot water released from industries are also considered as water polluting factors. Heavy metals,

fertilisers, pesticides alkalies, phenols, detergents, ammonia and cyanide are the common toxic contaminants (James, 1990). Not only the presence of toxic chemicals, waste waters comprising of large quantity of decomposing organic content, result in depletion of dissolved oxygen by microbial activity leading to the death of aquatic organisms (Crathorne and Dobbs, 1990; Zhu *et al*., 2011). Nitrogen and phosphorus reaching through urban sewage, agricultural run-off as well as atmospheric deposition eventually degrade aquatic ecosystems through phenomenon like algal blooming (Carpenter *et al*., 1998; Anderson *et al*., 2002). Persistent organic pollutants like polychlorinated biphenyls may act as endocrine disruptors and interrupt normal physiological mechanisms in aquatic vertebrates like fishes (Jones and De Voogt, 1999; Baldigo *et al*., 2006). Thus, various pollutants from point and non-point sources degrade the quality of environment and destabilize the balance of aquatic ecosystems.

Heavy metals are one of the major pollutants of aquatic ecosystems all over the world. Heavy metals are used by men for centuries for various purposes. Heavy metals have multiple applications in a wide range of human affairs resulting in their extensive dispersal all over the world and the raising of serious environmental concerns (Tchounwou *et al*., 2012). Often, they reach aquatic environment disrupting normal life processes of organisms. Factors like bulk production, environmental leakage during usage, toxicity, dispersion tendency, persistence and bioaccumulation are critical in making a chemical compound into a serious pollution hazard (Ali *et al*., 2013; Ali and Khan, 2017). With persistence and high toxicity, heavy metals are of great concern among various pollutants. Due to the non-biodegradable nature, heavy metals persist forever in the environments they are released (Mohammed *et al*., 2011). Significance of heavy metal threats is elevated as we consider the fact that many of them have the property of bioaccumulation and biomagnification as they move up in

food chains. Although heavy metals are naturally found in the Earth's crust, human activities cause increased release of these metals. Subsequently, elevated concentrations of heavy metals occur in environment resulting in detrimental effects on biota (Mohammed *et al*., 2011). Even the natural emissions of heavy metals may impair living organisms, leading to extinctions. Sanei *et al*. (2012) hypothesise that massive Permian extinctions are linked to the large scale mercury release from volcanic eruptions.

Prior to 1950's there were little scientific concern regarding the dispersal and toxicological effects of metals, although there were some early aquatic toxicological studies on metals. Massive flow in aquatic toxicological research simultaneously occurred by the establishment national environmental protection agencies and efforts to set water quality guidelines for contaminants by 1970's (Wood, 2011). Decreasing mobility, toxicity and bioavailability should be the major approaches of heavy metal control as they cannot be biodegraded (Mohammed *et al*., 2011).

According to Järup (2003), there is no specific definition for a heavy metal and those with a specific density above 5 g/cm^3 are generally considered as heavy metals. Heavy metals like mercury, lead, cadmium, arsenic and chromium are systemic toxicants inducing multiple organ damage even under lower level exposure and have high priority among pollutant heavy metals due to their high level of toxicity (Tchounwou *et al*., 2012). Some heavy metals like zinc, copper, iron, manganese and cobalt are essential for plants and animals as micronutrients in low concentrations (Rengel, 2004; Alloway, 2013). Even essential heavy metals act as toxins at elevated concentrations by the synthesis of complex compounds in the cell (Mohammed *et al*., 2011). Mercury and cadmium are typical metals among hazardous heavy metals, as

both of them pose serious issues of food contamination as well as environmental pollution (Tchounwou *et al.,* 2012). These two non-essential metals do not have any known biological role, where as they impart various toxicological effects on the normal physiology of organisms (Landis and Yu, 2003; Jaishankar *et al*., 2014).

Among aquatic pollutants, mercury is very hazardous as it is highly toxic as well as persistent. Properties of bioaccumulation and biomagnification make mercury more hazardous to the aquatic ecosystem. Global mercury emissions have natural as well as anthropogenic sources. Vast amounts of anthropogenic mercury have been accumulated in ecosystems, which is only undergoing a slow removal by biogeochemical cycles. Human activities make a large contribution to the atmospheric pools of mercury by releasing over 2,300 tonnes each year into the atmosphere. Urban, agricultural and industrial discharges, mining, combustion of fossil fuels as well as erosion and atmospheric deposition contribute to mercury contamination of aquatic ecosystems (Wang *et al.,* 2004). Coal combustion is the largest source contributing almost half of the total release of mercury (Pacyna *et al*., 2010).

Among the heavy metal pollutants, mercury is more dangerous being a neurotoxin and it may be converted to a more potent biological form, methyl mercury. Xenobiotic metals like mercury often disrupt normal physiology and homeostasis of organisms. Considerable reduction in primary anthropogenic emissions can only maintain oceanic mercury concentrations at present-day levels (Amos *et al*., 2013). Even if anthropogenic emissions are not rising, future deposition of mercury will be increased due to the already accumulated load of mercury. Rigorous scientific investigation of the sources, consequences and remediation of environmental mercury is necessary (Wiener, 2013).

Cadmium is a widespread non-essential metal raising environmental concern (McGeer *et al*., 2012). Cadmium occurs as a uniformly distributed rare element in the most abundant rocks of the Earth's crust (Hussain *et al*., 2017). Ever since men begin to produce metals from ores containing cadmium, environment became polluted with cadmium. Environmental cadmium load is further worsened during the last century by the use cadmium in various industrial products (Tchounwou *et al*., 2012). According to Järup, (2003) instead of recycling, cadmium containing products are often dumped with household waste which attributed the dramatic increase of cadmium emissions during the 20th century. Cadmium enters aquatic environment through atmospheric deposition following release from fossil fuel combustion, fertilisers, zinc mining and the manufacture of cadmium-containing articles (Muntau and Baudo, 1992; Bennet-Chambers *et al.,* 1999 and Pinot *et al.,* 2000).

Cadmium is a readily bioaccumulating toxic metal in aquatic organisms and it occurs in nearly all tissues. Cadmium has binding affinity similarities with nutritionally required elements like zinc, copper and calcium, resulting in cadmium protein interactions (McGeer *et al*., 2012). Cadmium is a heavy metal with no known biological function and inflicts toxic effect on the normal physiological functioning of the organisms. Cadmium is a ubiquitous heavy metal which poses great potential hazard to humans and to the wildlife as its uses are rising (Volesky and Holan, 1995; Tchounwou *et al*., 2012). Although it is previously established that cadmium can affect the reproduction of fish populations through gonadal bioaccumulation and endocrine disruption, a few studies only have investigated the non-lethal interaction of cadmium in fish. (Tilton *et al*., 2003).

Reproduction is one of the fundamental processes of life which ensures the continuity of the species. Any environmental factor which impairs reproduction directly or indirectly will contribute to species extinction. The negative impact of these factors may cause sterility, reduction in quality as well as quantity of progeny and even second generation infertility. Dichlorodiphenyltrichloroethane (DDT) induced reproductive failure contributed to the decline of populations and extinction risk in birds (Fry, 1995; Nakamaru *et al*., 2002). Reproductive failure linked to organochlorines in marine mammals has been reporting since 1970s (O'hara and O'Shea, 2001). Fishes are exposed to man-made chemicals in their environment, although many of them are not in high concentrations to cause sudden mortality. Still, harmful sublethal effects of these chemicals can potentially dwindle their populations (Hamilton *et al*., 2015). Environmental contaminants which induce toxic effects on reproduction directly influence the acceleration of population decline in organisms (CSTEE, 1999; Kamata, 2006; Lemos, 2010).

Although responses to various kind of stress may differ, energetic expenses are a common feature of all stress combating mechanisms (Schreck, 2010). Mechanisms to cope with stress affect reproductive fitness and stress physiology is closely linked to maturation and spawning (Schreck *et al*., 2001). Response to stress onset a set of events leading to the production of hormones like catecholamines and the elevation in stress hormones in turn physiologically alter organ systems (Schreck, 2010). Morgan *et al*. (1999) investigated the impact of stress in spawning cod, *Gadus morhua* and found out that compared to control fishes, plasma cortisol levels were elevated in stressed fish. Stressed fishes exhibited alterations in courtship sequence and more frequently produced abnormal larvae. Although successful spawning occurred under stressed conditions, reproductive output was likely to be affected.

Contreras-Sanchez *et al*. (1998) observed that, stress in rain bow trout, *Oncorhynchus mykiss* affected relative fecundity and ovulation timing, although growth of the juveniles and disease resistance were unaffected. Tolerance to stress differs with various taxa of fish, so that impacts on reproduction also will be divergent. Information regarding the physiological responses of a species to stress helps in developing strategies to manage its consequences (Schreck *et al*., 2001).

The major classes of toxic chemicals of concern for fishes are metals, chlorine, cyanides, ammonia, detergents, acids, pesticides, polychlorinated biphenyls, petroleum hydrocarbons, pulp mill effluents and other miscellaneous chemicals (Heath, 1995). Impacts of xenobiotics on fish reproduction are either by the direct effect on the development and viability of gametes or indirectly by disrupting the reproductive endocrine balance (Kime, 1999). There are several mechanisms for the effect of pollutants on fish reproduction, including endocrine disruption, interfering in egg formation, behavioural changes, damaging reproductive tissues and malformation of embryos (Sumpter, 1997; Scott and Sloman 2004). Xenobiotics with endocrine disrupting properties may disrupt hormonal pathways causing feminization of male fish or decreased fertility in female fish (Arcand-Hoy and Benson, 1998; Caballero-Gallardo *et al*., 2016).

Fish short term reproductive assay evaluates the potential of compounds to interfere in the normal reproductive function of a model fish by assessing a variety of endpoints. Endpoints like fecundity, fertilisation success, gonadosomatic index (GSI), gonadal histopathology or vitellogenin may indicate disruption of hypothalamic-pituitary-gonadal axis of fishes (US Environmental Protection Agency, 2009). Species specific differences in the reproductive strategies cause variations in these parameters, thus substantial knowledge on the natural variabilities is necessary for assessing toxic

impacts on reproduction (Segner, 2011). Comparative toxicity data related to reproductive endpoints are limited for most taxonomic classes of vertebrates and such information aid in recognising species sensitive to metals and populations at risk (Wiener, 2013).

Wild fishes are a group of organisms worst affected by aquatic pollution. Presence of toxicants in the environment are a significant aspect affecting fish health, due to their intimate contact with aquatic medium around them through gills and skin surface. Although fish diseases are localised in nature, some pathological conditions like liver tumors present in demersal fish existing in polluted waters related to the release of toxic chemicals raises concern among scientists (Bucke, 1993; Blazer *et al*., 2018). While lower levels of pollutant discharge cause bioaccumulation of toxicants leading to consequences in long time, enormous discharges result in large scale fish kills (Austin, 1998; Abowel and Sikoki, 2005). Being the species under high risk of water pollution and its presence in almost all aquatic ecosystems makes fishes ideal candidates of toxicity testing of contaminants relevant to aquatic environment.

Even toxicant exposure given to the test fishes under controlled conditions of laboratory setting can be compared to pollution subjected by wild fishes in natural habitats (Kime, 1999; Bambino and Chu, 2017). Relevance of fishes as an important part of human diet and the possibility of human health impacts through toxicants accumulated in fish species add to their significance as potential targets of environmental studies (Katagi, 2010). Compared to rodents, fishes are more effortless to maintain and easily available for experimental purposes. They also allow the possibility of increased number of replicates in testing various parameters than many other vertebrate test organisms.

Quality of water has profound importance in the health of human beings as well as sustainability of ecosystems. Establishing water quality standards are essential prior to formulating laws and regulations regarding pollution control. Various life cycle stages of fishes have extensive application to toxicity assays for establishing water quality criteria essential for the sustenance of aquatic biota (Javed and Usmani, 2017).

Aquatic invertebrates are not ideally suitable for monitoring endocrine disruptors of the environment, even though they are commonly used for water quality monitoring programmes (Kime, 1999). Physiological impacts of endocrine disrupting chemicals in fishes are evidenced globally and widely described among them were in reproductive dysfunction (Söffker and Tyler, 2012). Availability of large sample sizes in terms of gametes, embryos and juveniles also enhances utility of fishes as test organisms in reproductive toxicity testing. Fertilisation and hatching rates are readily determinable and developmental defects of offspring are effortlessly monitored for fishes (Kime, 1999). Thus, fishes are very suitable test group for studying the impact of aquatic pollutants on the reproductive function of organisms. The present study investigates the effects of heavy metals mercury and cadmium in the reproduction of native fresh water fish, *Rasbora dandia*.

As the present investigation is focussed on the toxic impact of heavy metals on fish reproduction, reproductive biology of the test fish, *R. dandia* is elucidated as part of the study. Acute toxicity studies are the essential data requirement for proceeding further investigations in ecotoxicological studies and median lethal concentration of mercury and cadmium is assessed. Bioaccumulation is a vicious property of heavy metals which exacerbate their impact in the ecosystem.

Bioaccumulation of mercury and cadmium in the gonads of test fish, *R. dandia* is studied in order to get an understanding of the nature of the accumulation of heavy metals in the gonads. Effect of heavy metals in the sexually mature gonads as well as its influence in the gonadal recrudescence of *R. dandia* is investigated in the present study. Toxic influence on the embryonic development impairs progeny quality and health of wild fish populations. The effect of heavy metals in the embryonic development of *R. dandia* is also included in the present study.

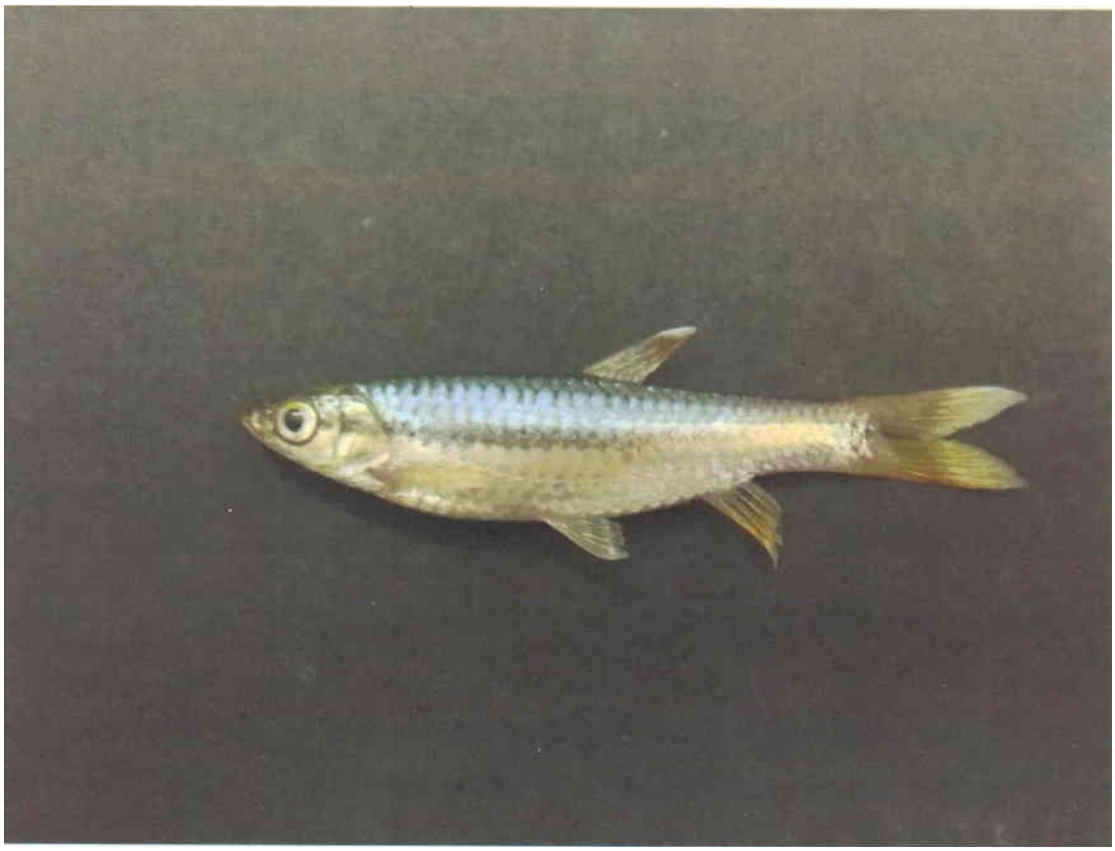
CHAPTER 2

TAXONOMY AND REPRODUCTIVE BIOLOGY

Introduction

Fishes are excellent model organisms which played crucial role in the development of several disciplines including environmental biology (Cossins and Crawford 2005). Along with the practical and technical benefits, a model organism should have generalised traits representing a vast group of organisms (Segner, 2009). Fishes as an experimental animal, possess many practical advantages like availability and easy maintenance in the laboratory conditions. A high level conservation is present in endocrine systems between fish and other vertebrates (Spitsbergen and Kent, 2003). Despite of a few limitations, bioaccumulation and biomarkers responses in fish are excellent tools of assessing the impact of environmental contaminants on aquatic ecosystems (van der Oost *et al*., 2003).

Cyprinids are the largest and ecologically diverse freshwater fish family in the world (Zardoya and Doadrio, 1999; Ney and Helfrich, 2009). Rasborins are one of the major species rich groups of Cyprinid subfamilies *Danioninae* and *Rasbora* is the most species richest genus among them (Liao *et al*., 2010). *R. dandia* is a small cyprinid fish found well distributed throughout the southern states of India like Kerala, Karnataka and Tamil Nadu as well as Sri Lanka (Silva *et al.,* 2010). The fish, *R*. *dandia* is selected for the present study, because it is sturdy enough to maintain and can be induced to breed in laboratory conditions.



Rasbora dandia

Phylum: Chordata

Class: Actinopterygii

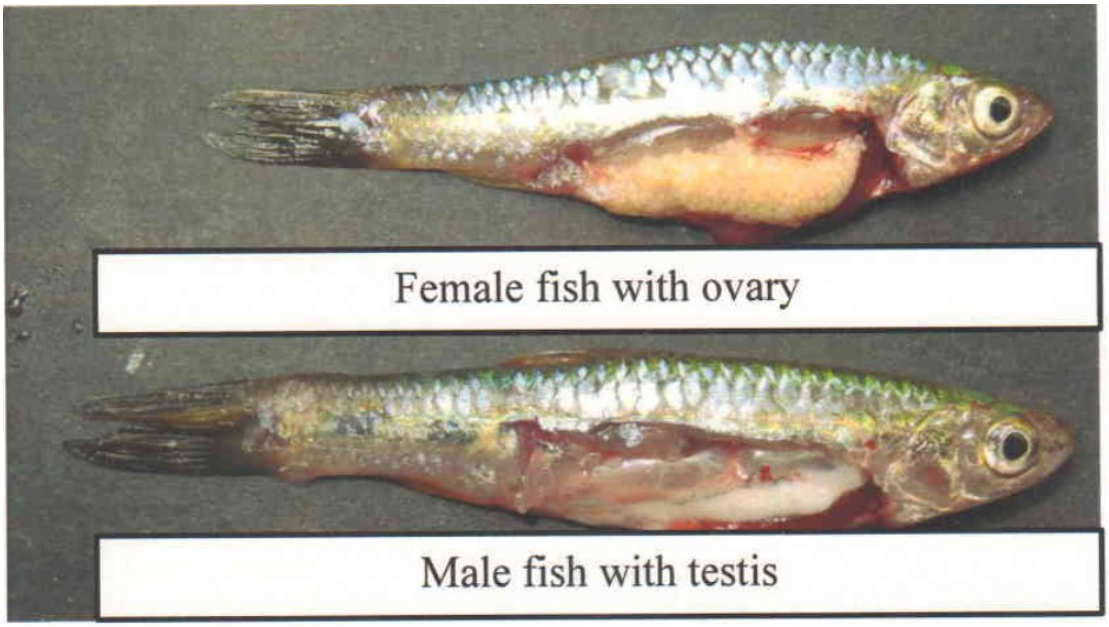
Order: Cypriniformes

Family: Cyprinidae

Sub family: Danioninae

Genus: *Rasbora*

Species: *dandia*



Male and female *R. dandia*

Large extent of comparative information obtainable from DNA sequence data has a major influence on taxonomy accelerating it into a more phylogenetic approach from simple descriptive approach (Harley, 2009). Majority of the animal diversity exhibit enough sequence diversity of mitochondrial genomes to permit the discrimination of closely related species (Herbert *et al*., 2003a).

Cytochrome-c oxidase is a complex metalloprotein in mitochondria playing a vital role in the respiratory chain catalysing the oxidation of ferrocytochrome c and reduction of dioxygen to water (Dalziel *et al*., 2006; Li *et al*., 2006). While small subunits of cytochrome *c* oxidases are synthesised by nucleus, three major core subunits I, II and II are encoded by mitochondrial genome in eukaryotes (Scott, 1995). CO1 (cytochrome c oxidase subunit I) is among mitochondrial genes with high rates of nucleotide substitution, and variation, despite of its conserved amino acid sequences (Mueller, 2006). According to Herbert *et al*. (2003b) COI has the potential to function as a global bioidentification system for animals and this system can provide a reliable solution to problem of species identification under fully developed condition. Silva *et al*. (2010) found that *R*. *dandia* is a cryptic species and separated *R*. *dandia* from *R*. *daniconius* based on morphological features. According to them, *R*. *dandia* is found well distributed throughout the southern states of India like Kerala, Karnataka and Tamil Nadu as well as Sri Lanka. Tang *et al*. (2010) pointed out that a much needed revision is required for the genus *Rasbora*. A detailed morphological analysis, especially the number of scales in the lateral line series has shown that fishes collected for the present study was akin to morphological description of *R*. *dandia* given by Silva *et al*. (2010). In order to confirm the taxonomic status of *R*. *dandia* with molecular evidence, COI study has been conducted.

Teleost fishes are the largest group among vertebrates occurring in almost all aquatic ecosystems. It is crucial to gain knowledge regarding maintenance, growth and reproduction while understanding the ecology of a fish species (Wootton, 1990). Teleost fishes adopt wide variety of reproductive strategies to adapt themselves to diverse aquatic environment. Among vertebrates, teleost present most remarkable reproductive diversity (Smith and Wootton, 2016). The fish reproduction varies in patterns of sexuality, gametogenesis, spawning, parental behaviour and response to environmental factors (Jalabert, 2005).

Attention to basic life history traits is essential in fisheries management and such information are often missing for newly exploited species (King and McFarlane, 2003). Reproduction is an important life history parameter to be considered while acquiring information of the ecology of any organism. Gaining knowledge of the reproductive biology of a species is a key concept in disseminating scientific knowledge in fisheries management as it plays major role in regulating productivity. It gives insight on the resilience of a fish species to commercial fishing exploitation as well as environmental disruptions from anthropogenic activities (Morgan, 2008). Reproductive biology of an organism has pivotal role in conservational biology as it provides information regarding captive breeding and biological control (Wikelski and Cooke, 2006). Sexual pattern, reproductive cycle, follicle development and spawning periodicity are important aspects of fish reproductive biology while determining precise management strategies for a species (Alonso-Fernández *et al*., 2011).

Teleost fishes are primarily dioecious, although hermaphroditism is present in a few species. Spermatozoa and eggs are produced by separate individuals and expelled it in to water for fertilization and development (Taborsky and Neat, 2010).

Among hermaphrodite fishes, synchronous hermaphroditism is rare while sequential hermaphroditism is common (Wootton and Smith, 2015). Majority of fishes adopts the strategy of external fertilization where the fusion of spermatozoa with eggs is facilitated in the aquatic environment. Mating behavior ensures species specificity in internally fertilising species while added mechanisms are utilized by externally fertilising fishes (Kinsey *et al.,* 2007). During internal fertilisation, spermatozoa get motile in the ovarian fluid and activate eggs in the ovary (Iwamatsu, 2000). In externally fertilising fishes, sperm activation is controlled by the variation of the internal osmolarity of sperm cells with those of the aquatic medium (Browne *et al.,* 2015). Fundamental structures of teleost gonads are similar despite of the complexity of diverse reproductive patterns among them (DeFalco and Capel 2009).

Testes are elongated and paired organ connected to the dorsal side of the body wall in majority of teleosts. In some species like poeciliids, testes are fused to appear as a single sac. Testes are open to outside via urogenital pore and connected caudally if occur in paired condition (Spadella *et al*., 2008; Uribe *et al*., 2014). Two types of internal structures of testes were identified in teleosts. In tubular pattern, lumen is absent and cysts move to the vas efferens from the blind end during spermatogenesis. Lobular testis has centrally located lumen accepting spermatozoa produced from stationary cysts along the lobule (Uribe et al., 2014). Germ cells undergo a series of structural and physiological changes progressing through stages like spermatogonia, spermatocytes, spermatids and spermatozoa. Histology of testis exhibit marked structural differences during various phases of reproductive cycle. Free spermatozoa present sparingly in the lumen during early spermatogenesis phase. During middle spermatogenesis a minor population of free spermatozoa occurs in the lumen. Most of

the tubules are filled with free sperms during late spermatogenesis (Uribe *et al*., 2014).

Generally, teleost ovaries are paired hollow structure although single ovary formed from the fusion of left and right ovaries do occur in some species. Ovaries are connected to the body wall by oviduct and ovulated eggs deposited out of the genital pore through oviduct (Iwamatsu, 2000). Ovaries vary significantly during various phases of reproductive cycle. Ovary weight may increase up to 40% of the total body weight in synchronous spawners. Weight of the ovary is lesser in asynchronous spawners and grows recurrently many times during spawning season with regular periodicity (Tyler and Sumpter, 1996). Teleost ovary includes oogonia, oocytes, follicle cells and supporting tissue along with nervous and vascular tissue (Yaron and Levavi-Sivan, 2011). Oogenesis, oocyte growth, cortical alveoli formation, vitellogenesis, maturation and ovulation are the essential pattern of oocyte development in teleost fishes (Tyler and Sumpter, 1996).

Mercy *et al.,* (2013) studied the reproductive biology of *Puntius denisonii,* which is an endemic fish from the Western Ghats of India. Marked sexual dimorphism was absent other than characteristic behavior and extended belly of the female. Fish spawns once in a year and was found to be from December to February in Iritti river. Kashyap *et al.* (2016) investigated the reproductive biology of freshwater murrel, *Channa punctatus*. Spawning season extends about four months from May to August as indicated by the GSI variation and presence of fishes with ripe gonads. Bruton (1979) studied the breeding biology of *Clarias gariepinus* of Lake Sibaya, South Africa. Gonadal recrudescence is connected with increasing photoperiod and temperature. Before spawning fishes make massive aggregation and

courtship occur after aggressive encounter among males. Spawning occurs during the night following heavy rain.

Reproductive biology of exotic *Tilapia rendalli* and indigenous *Puntius sarana* in an ancient man-made lake in Sri Lanka was carried out by Chandrasoma and Silva (1981). Female to male ratio was considerably different among two species which is related to migration and spawning behavior. Males were dominant in *T. rendalli* while the opposite pattern was observed in *P. sarana*. Both species of fishes were breeding throughout the year and marked peaks of breeding associated with rainy seasons were found in *P. sarana*. Ali and Kadir (1996) studied the breeding cycle of tropical cyprinid, *Thynnichthys thynnoides* from Chenderoh reservoir in Malaysia. Breeding cycles were coincided with increase in reservoir water level causing littoral zone flooding. Five gonad development stages were observed in females while four stages were exhibited by male fishes. *T. thynnoides* was found to be a total spawner but batches of ripe eggs might be spawned over an extended period. Potts *et al.* (2005) investigated the reproductive biology of cyprinid, *Labeo umbratus* in small South African reservoirs. Reproductive strategies like high fecundity, prolonged spawning season and rapid larval development of *L. umbratus* seems to be well adapted to varying environment of small reservoirs. They also found that gonadosomatic index (GSI) was related to water temperature as well as day length in the fish.

Muchlisin *et al.* (2010) studied the spawning seasons of *Rasbora tawarensis* from Lake Laut Tawar, Aceh Province, Indonesia. *R. tawarensis* spawns three times in a year with peak reproductive period in September. The fish is found to be a group synchronous spawner and predominant sex is female. Guraya *et al*. (1975) conducted

the morphological and histological studies of the reproductive cycle of ovary in the cat fish, *Mystus tengara*. The fish breeds once in a year and spawning is initiated by monsoon rains. Seven phases were identified in the ovarian cycle based on morphological changes, developmental stages of oocytes and GSI.

The annual ovarian cycle of the flounder, *Pleuronectes flesus* was investigated by Janssen *et al*. (1995). Oocytes were developed in a group synchronous process and occurrence of two different batches of oocytes in primary growth phase was found. One among these groups transform in to vitellogenic stage by the end of summer and second group remain in primary growth stage. Seven stages of oocyte development were distinguished other than the oogonia. They were perinucleolus, cortical alveoli phase, early vitellogenesis, advanced vitellogenesis, final maturation, ovulation, spawning and a post-spawning phase.

Sharma and Bhat (2015) studied annual reproductive cycle of male Rainbow Trout, *O. mykiss*. Four maturational stages were identified in the testis including resting stage, maturation stage, mature stage and regression stage. GSI was highest during mature phase and lowest during resting phase. Primary spermatogonia, secondary spermatogonia, spermatocytes, spermatids and spermatozoa are the various stages of germ cells identified from histological studies. Largest among them were primary spermatogonia which occur in the periphery of testis lobules. Germ cells of various stages were visible in maturing phase of testis and lobules were filled with spermatozoa only in mature phase.

Reproductive cycle of perciform fish, *Cichlasoma dimerus* under laboratory conditions was studied by Vázquez *et al.* (2012). *C. dimerus* has lobular testis with cystic type of spermatogenesis. Multiple spawning occurred throughout the

year with high reproductive activity from September to March. Regressed, early maturation, mid maturation, late maturation and regression were the major stages of testicular recrudescence identified. But only four stages occurred during season of high reproductive activity as regression stage was absent then.

There are several physiological processes during fish spawning like germinal vesicle breakdown, ovulation, spermiation and sperm release. These physiological processes are cued by various environmental factors including temperature, chemical parameters of water, rain fall and social factors (Pankhurst and Munday 2011). Under captivity, fishes exhibit reproductive dysfunction due to failure in physiological processes associated with spawning. Captive fishes are not exposed to particular environmental conditions of spawning ground which prevents endocrine secretions necessary for spawning (Zohar and Mylonas, 2001; Mylonas *et al*., 2010). Application of homogenized pituitary extract was the earliest method of inducing spawning in captive fishes. Later, potential synthetic analogues of gonadotropin-releasing hormone to overcome reproductive failure of captive brood stocks aided in controlling fish reproduction in aquaculture (Harvey and Carolsfeld, 1993; Zohar and Mylonas, 2001).

Reproductive endpoints are valuable markers for ecosystem monitoring programs. Fish reproduction is an ecologically significant indicator of endocrine disruption especially of estrogenic action as there is strong relationship between reproductive impairment due to endocrine disrupting compounds and succeeding population level effects (Arcand-Hoy and Benson, 1998). Data regarding the pattern of normal reproductive cycles of organism are useful in investigating altered reproductive conditions under toxicant impact in polluted ecosystems. Janssen *et al*.

(1997) observed premature vitellogenesis causing high number of vitellogenic oocytes in the ovary of flounder, *Platichthys flesus* from polluted environment during previtellogenic period of the annual reproductive cycle. Descriptions of normal annual cycle of the ovaries are important as they serve as control in toxicological studies assessing the impact of environmental pollution (Janssen *et al*., 1997).

Rokade *et al*. (2006) compared efficiency of Ovaprim which contains analogue of Salmon GnRH and dopamine inhibitor with carp pituitary extract in the induced breeding of major carp, *Cirrhina mrigala*. Spawning latency period was 16 hours under carp pituitary extract injection, while spawning occurred in 9 hours on administering Ovaprim. Egg count as well as fertilization rate was higher in Ovaprim injected fishes indicating high fertility in fishes compared to carp pituitary extract application. Mollah and Tan (1983) induced ovulation in catfish *Clarias macrocephalus* using human chorionic gonadotropin (HCG) and acetone-dried pituitaries from sexually mature catfish homogenized in normal saline. HCG (2 IU/gm of body weight) induced ovulation in 95% of fishes while it was only 88% among pituitary extract (3.5 mg/100 g body weight) injected fishes.

Ingram and Rimmer (1993) subjected endangered trout cod, *Maccullochella macquariensis*, to captive breeding for the production of fry to reintroduce into the wild. Although gonadal maturation occurred in fishes maintained in earthen ponds during spring season, spawning did not follow. Ovulation was successfully induced by injecting 1000-3000 IU/kg of HCG and spawning was higher when fishes induced bred when water temperature was lower than 16°C. Induction of oocyte maturation in white croaker, *Micropogonias furnieri* using HCG was demonstrated by Gracia-Alonso and Vizziano (2004). Induced breeding of *P. sarana* was conducted under

captivity by Udit *et al*. (2014). Brood stock was acclimatized and maintained with balanced fish diet along with tubifex worms. Fishes were successfully induced bred with Ovatide with dosage of 0.2 ml per male (180 g) and 0.3 ml per female (232-240 g). Reproductive biology of *R. dandia* was investigated in the present study along with induced breeding and the study of embryonic stages of the fish, which was also been carried out.

Materials and methods

Collection and maintenance of test organism

The fish, *R. dandia* was collected from fresh water bodies of Manalur (latitude: 10.4855^{o} N; longitude 76.1030^{o} E) which is a part of North Kole wetland of Thrissur district, Kerala. The collected fishes were transported immediately to the laboratory and transferred gently to large aquarium tanks filled with non chlorinated fresh pond water received through water inlet system. The following physicochemical parameters of the water in all aquaria were maintained throughout the experimental period: temperature, 26^{o}C to 28^{o}C, pH 6.80 to 7.00, dissolved oxygen 6.80 ppm, and total hardness 36.93 ppm. The fishes were acclimatized to laboratory conditions for fourteen days before transferring the fishes to small aquaria for experimental purpose. All the aquaria were well aerated through a mechanical system. The fishes were fed daily with standard fish feed.

Gene sequencing and phylogeny analysis

The genomic DNA of *R. dandia* was extracted using GeNei Ultrapure Mammalian Genomic DNA Prep Kit (Genei Laboratories Pvt Ltd, Bangalore). Extraction of DNA was carried out according to the manufacturer's instructions. 2.00 ng of genomic DNA was amplified for CO1 gene in a thermocycler (Eppendorf,

Germany) utilising the forward primer with DNA sequence 5'-ATTCAACCAATCATAAAGATATTGG-3'and reverse primer with DNA sequence 5'-CTCCACCAGCAGGATCAAAA-3'. The PCR reaction mixture comprised of 2 ng of genomic DNA (1 µl), 1 µl each forward and reverse primers at a concentration of 10 µM, 2 µl of dNTPs (2 mM), 2 µl 10X reaction buffer, 0.20 µl Taq polymerase (5 U/µl) and 12.8 µl H_2O. The initial denaturation step was of three minutes at 95^0 C, which was followed by 35 cycles of ten seconds at 95^0 C, ten seconds at 50^0 C and 45 seconds at 72^0 C and had a final extension ended for 3 minutes at 72^0 C for 3 minutes (Sambrook and Russel, 2001). Column purification of the PCR product was done using Mo Bio UltraClean PCR Clean-up Kit (Mo Bio Laboratories, Inc. California). The purified nucleic acid was sequenced with Sanger's sequencing method from both ends using the forward and reverse primers. The sequences reported from DNA sequencing facility was edited by trimming off both the forward primer sequence and reverse primer sequence off. The primer sequences were trimmed from the forward and reverse sequences were acquired. The sequences which were aligned with Clustal W (Thompson *et al*., 1994), a multiple sequence alignment programme. The resultant consensus nucleotide was checked for sequence similarities, using BLAST programme of NCBI (www.ncbi.nlm.nih.gov).

For standalone phylogeny analysis, closely related sequences were downloaded from the NCBI database. In order to make a rooted tree, an out-group of the species was also provided by adding an Operational Taxonomic Units (OTU) namely CO1 sequence from a specimen of *Danio rerio,* which was downloaded from the GenBank was used in the alignment. A list of downloaded sequences together with their author reference is provided in the Table 2.1. The downloaded sequences were arranged in the sequence alignment program, Bioedit (Hall, 1999)**.** The

alignment was then arranged using Clustal W operation. Gaps and missing data were excluded from the alignment using the editing facility in the program. The edited alignment file was then opened in the phylogeny analysis program MEGA (Version 4) (Tamura *et al*., 2007).

Table 2.1: Table showing author reference, accession number and species name of CO1 sequences used to construct phylogeny in the present work.

Sl. No.	**Authorship**	**Accession Number**	**Species**
1	Priya *et al.* 2015 (Present work)	KP742982.1	*Rasbora dandia*
2	Kalyankar *et al.* 2012a	JX260962.1	*Rasbora wilpita*
3	Kalyankar *et al.* 2012b	JX887606.1	*Rasbora labiosa*
4	Kusuma *et. al.* 2016	LC130777.1	*Rasbora argyrotaenia*
5	Kusuma *et. al.* 2016	LC130651.1	*Rasbora lateristriata*
6	Britz *et al.* 2009	FJ753499.1	*Rasbora daniconius*
7	Van Der Walt *et al.* 2016	KU568810.1	*Danio rerio*

List of accession numbers and title of NCBI data.

JX887606 *R. labiosa* voucher RLKW01 cytochrome oxidase subunit 1 (CO1) gene, partial cds; mitochondrial. (Kalyankar V.B., Khedkar G.D., Lutzky S. and David L., 2012b)

JX260962 *R. wilpita* voucher PMUM01 cytochrome oxidase subunit 1 (CO1) gene, partial cds; mitochondrial. (Kalyankar V.B., Khedkar G.D., David L. and Lutzky S., 2012a)

KP742982.1 *R. dandia* cytochrome c oxidase subunit I (CO1) gene, partial cds; mitochondrial. (Priya R., Arunkumar K.M., Kottickal L.V. and Kuzhivelil B.T., 2015)

FJ753499.1 *R. daniconius* isolate LR1668 cytochrome oxidase subunit 1 (CO1) gene, partial cds; mitochondrial (Britz R., Conway K.W. and Ruber L., 2009)

LC130777 *R. argyrotaenia* mitochondrial CO1 gene for cytochrome c oxidase subunit I, partial cds, isolate: Cijenuk 5. (Kusuma W.E., Ratmuangkhwang S. and Kumazawa Y., 2016)

LC130651 *R. lateristriata* mitochondrial CO1 gene for cytochrome c oxidase subunit I, partial cds, isolate: Tegal 7. (Kusuma W.E., Ratmuangkhwang S. and Kumazawa Y., 2016).

KU568810.1 *D. rerio* voucher KW11T169 cytochrome oxidase subunit 1 (CO1) gene, partial cds; mitochondrial (Van Der Walt K. A., Makinen T., Weyl O., Collins R. and Swartz E., 2016).

Histological preparation

To prepare histological slides of testis, twenty acclimatized gravid male fishes were kept in aerated aquaria. Five male fishes were selected at random and the testes were dissected out carefully, washed and placed in 10% formalin for fixation. The histological slides were prepared according to Alonso-Fernádez *et al*. (2011). Briefly, the testes were dehydrated in ethyl alcohol and cleared in xylene. Alcohol grades of 30%, 50%, 70%, 96% and 100% were used serially for dehydration. Then, testes were infiltered with paraffin wax and embedded in paraffin blocks. 1-3 µm specimens' sections were taken using rotary microtome and stained using hematoxylin (Merck, India) and eosin (Merck, India) (H & E).

As in the case of male fishes, from twenty acclimatized gravid female fishes, five fishes were selected at random and ovaries were dissected out from them carefully. These ovaries were washed and placed in Bouin's fluid for fixation. Histological slides of ovary were prepared according to Alonso-Fernádez *et al*. (2011). The ovaries were dehydrated in ethanol and cleared in methyl benzoate. For dehydration, alcohol grades of 30%, 50%, 70%, 90% and 96% were used serially.

After clearing, the specimens were infiltered with paraffin wax and embedded in in paraffin blocks. 6 µm sections of ovary were taken using rotary microtome and stained using hematoxylin and eosin (H & E).

Stained sections of gonad were analyzed under light microscope (Leica Microsystems, Germany) and photographed using Leica image capture software, Leica Las EZ.

Maturity stages of the Gonad

The teleost fish, *R. dandia* were selected at random from the stock in the laboratory in each month and dissected out the testes and ovaries carefully. The gonads were washed and thoroughly examined for the morphological characteristics described in table 2.2 and recorded. The maturity stages of the fish were classified according to Teji (2010) as immature, maturing, mature, ripe and spent.

Annual Breeding Cycle, Stages of Oocyte and Spermatocyte Development

To study the annual breeding cycle, total of 100 male and female fishes were collected each month for a period of three years, acclimatized and maintained in the laboratory as described earlier. Ten male and ten female fishes were selected at random and reproductive stage of the fishes were studied. Number of ripe fishes in each month was noted and recorded. The diameter of the egg and spermatocyte was measured to the nearest value using micrometer (Teji, 2010) Further, to determine the reproductive stage of the fish, the excavated gonads were histologically studied as described earlier. The female reproductive cycle was defined by follicular development stages in the ovaries and the male reproductive cycle was defined by progress of spermatogenesis stages in the testis (Ki and Lee, 2018).

Induced Breeding

During monsoon season, *R. dandia* were collected, transported as described earlier and acclimatized to the laboratory conditions. Ten gravid males and ten gravid females were selected at random and kept in small aquarium. Ten such replicates were maintained. Gravid male which oozes out milt and gravid female which release eggs on slight pressure on the abdomen were selected for breeding (Haniffa and Sridhar, 2002). As the fish *R. dandia* did not breed in captivity, they were administered with hormone injection and kept in pairs in well aerated glass aquaria. They were fed with standard fish feed.

Various doses of human chorionic gonadotropin (HCG) hormone (Bharat Serums and Vaccines Ltd, India) (5000 IU/ml) and Ovaprim (Syndel Laboratories, Canada) were tested in gravid male and female fishes for finding out the optimum dose for breeding under laboratory conditions. Ovaprim contains salmon gonadotropin releasing hormone analogue (sGnRHa) (20 µg/ml) and domperidone (10 µg/ml). HCG of 1000 IU, 2000 IU, 3000 IU, 4000 IU and 5000 IU per kg body weight of fish were administered to male fishes. The female fishes were administered with HCG doses of 2000 IU, 6000 IU, 8000 IU and 10,000 IU per kg body weight of fish (Di Maggio *et al*., 2014).

The required dose of Ovaprim to be administered to the fish was calculated according to Yanog *et al*. (2009). Injected doses of Ovaprim to the fish, *R. dandia* were 0.50 ml, 1.00 ml. 2.00 ml and 2.50 ml per kg body weight. All the injections were administered to the fish intramuscularly at the dorsal region of the caudal peduncle using a fine hypodermic needle. The fishes were then continuously observed.

Egg stripping and fertilization were carried out according to Nayak *et al*. (2001). The fishes were cleaned and the eggs were stripped by applying gentle pressure on the abdomen. The eggs were collected in plastic trays. The sperms were obtained from the male fishes by stripping as done in the case of female fishes. The freshly collected sperms and eggs were mixed in a plastic tray using bird feather for 2-5 minutes and then water is added slowly into the tray.

The fertilized eggs were washed and continuously observed for embryonic development. Embryonic stages were identified and photographed using a stereoscopic microscope (Leica M 205 C. Germany with the help of the software Leica application suite version 4.3.0). The embryonic stages in the fish were identified according to the description provided by Kimmel *et al*. (1995).

Results

CO1 Gene sequence and phylogeny analysis

The PCR of the CO1 gene fragment of *R. dandia* yielded a single product of 658 bp. The BLAST search using the sequence revealed that the sequence obtained in this study was novel (GenBank Accession No. KP742982).

CO1 Gene sequence of *R. dandia*

```
TACCCTTTATCTCGTATTCGGTGCCTGAGCTGGAATAGTTGGAACCGCCCTTAGTCTTCT
CATTCGTGCTGAACTTAGCCAACCGGGATCACTACTAGGAGACGATCAAATTTATAATGT
CATTGTAACTGCTCATGCTTTTGTAATGATTTTCTTTATAGTTATACCAATACTTATCGG
AGGATTTGGGAACTGATTGGTCCCACTGATAATTGGGGCACCAGACATAGCATTCCCCCG
AATAAACAACATAAGCTTTTGACTTCTCCCCCCATCATTCCTTCTTTTATTAGCCTCCTC
TGGTGTTGAAGCAGGTGCCGGGACAGGATGAACAGTTTATCCACCACTTGCAGGAAACCT
CGCCCACGCAGGAGCATCAGTAGACCTAACAATTTTCTCTCTCCACTTAGCAGGTGTGTC
ATCAATCTTAGGGGCTATTAATTTCATTACAACAATTATTAATATGAAGCCTCCAGCCAT
TACTCAATATCAAACTCCTTTATTTGTCTGAGCCGTACTAGTAACAGCCGTGCTTCTACT
ACTATCCCTACCAGTCCTAGCTGCTGGAATCACAATGCTCCTTACAGATCGAAACCTTAA
CACTACTTTCTTTGACCCGGCTGGAGGGGGAGATCCTATTCTTTATCAACACTTATTC
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A Basic Local Alignment Search performed using the tool BLAST available in the NCBI website showed that the closest relatives of the specimen were appeared

in at least two taxonomic identities *i. e., R. daniconius and R. dandia.* Other related species were *R. labiosa and R. wilpita* which are distributed in Sri Lanka. The phylogeny analysis was thus conducted using the available DNA sequence data of mitochondrial CO1 gene, downloaded from NCBI. Phylogeny analysis using DNA distance and bootstrap statistical operations provided a tree diagram in which the phylogenetic relationship of the species is well represented in the figure 2.1.

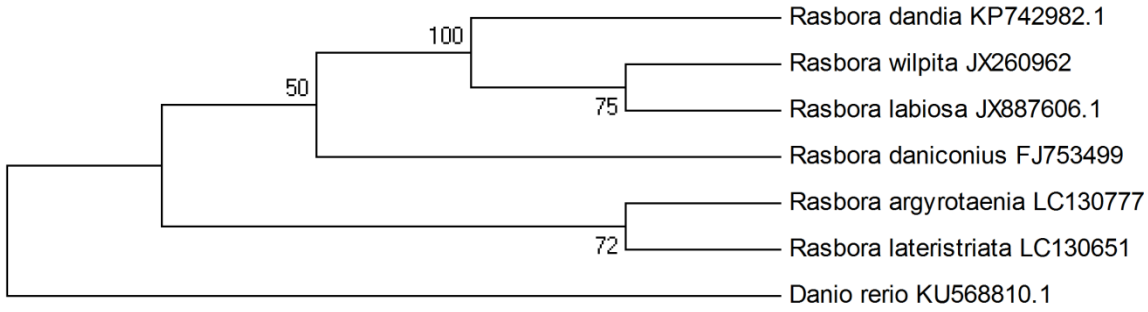


Figure: 2.1, Phylogram showing evolutionary relationships of seven taxa

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The bootstrap consensus tree (Felsenstein, 1985) inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analysed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura, *et al.,* 2004) and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There was a total of 512 positions in the final dataset. Phylogenetic analyses were conducted in MEGA Version 4 (Tamura *et al.,* 2007).

Reproductive biology

Sexual dimorphism

Prominent features of sexual dimorphism are absent in *R. dandia*. Females are often larger while males are slender and small. Abdomen of females are round and plump especially during the sexually mature stage.

Annual breeding cycle

R. dandia is a seasonal breeder. Breeding is associated with south west monsoon. Breeding in *R. dandia* start by the onset of south west monsoon in June. Sexually mature specimens obtained until November indicates a prolonged breeding season extending from June to November. But peak season of spawning is focussed around June as indicated by the presence of highest proportion of ripe fishes in the month (figure 2.2).

Table 2.2: Classification of maturity stages of the gonads of *R. dandia*

Maturity stage of gonad	Characteristics of testes	Characteristics of ovaries
Immature	Appear as inconspicuous transparent thread like structure.	Appear as thin, transparent thread like structure with a few black dots on it. Eggs were not visible unless under microscopic examination.
Maturing	Begin to thicken with opaque milky appearance.	Start to enlarge with a gel like appearance. A few ova start becoming visible like scattered yellowish spots.
Mature	Increase in volume and blood vessels become visible. Milt starting exuding out of testis sparingly.	Gains size with opaque yellow colour. Ovaries fill up to half the volume of body cavity. Large transverse blood vessel appears branching laterally over the ovary.
Ripe	Enlarge and occupy half of the body cavity. Large amount of milt start oozing out on slight pressure on abdomen.	Increase in volume occupying $3/4^{th}$ of the body cavity. Abdomen become distended and extrudes eggs on slight pressure.
Spent	Diminish in size and appear white without milt secretion.	Appear flaccid with brownish yellow colour. Few remnant eggs are visible dispersed throughout the ovary.

Stages of oocyte development

Oogonium: Oogonia appear as small cluster of weakly basophilic cells (figure: 2.3). They are roughly spherical cells with diameter ranging from 10.06 µm-15.64 µm (12.64 ± 1.73 µm). Nucleus is spherical with diameter ranging from 5.53 µm-8.91 µm (7.15 ± 0.91 µm). Oogonial cells are conspicuous in immature ovaries only.

Chromatin nucleolus Stage: Oocytes in this phase are round or oval, basophilic cells (figure: 2.4). Diameter of cells ranges from 21.48 µm-27.74 µm (24.75 ± 1.86 µm). Large spherical nucleus has a diameter ranging from 8.9 µm-16.14 µm (13.06 ± 2.24 µm).

Early perinucleolus Stage: Oocytes appear as oblong basophilic cells with diameter ranging from 28.03 µm-67.72 µm (48.61 ± 13.29 µm) (figure 2.6). Nucleus is slightly eccentric with diameter ranging from 13.36 µm-40.16 µm (24.65 ± 8.59 µm). Several nucleoli are visible in the nucleus (figure 2.5).

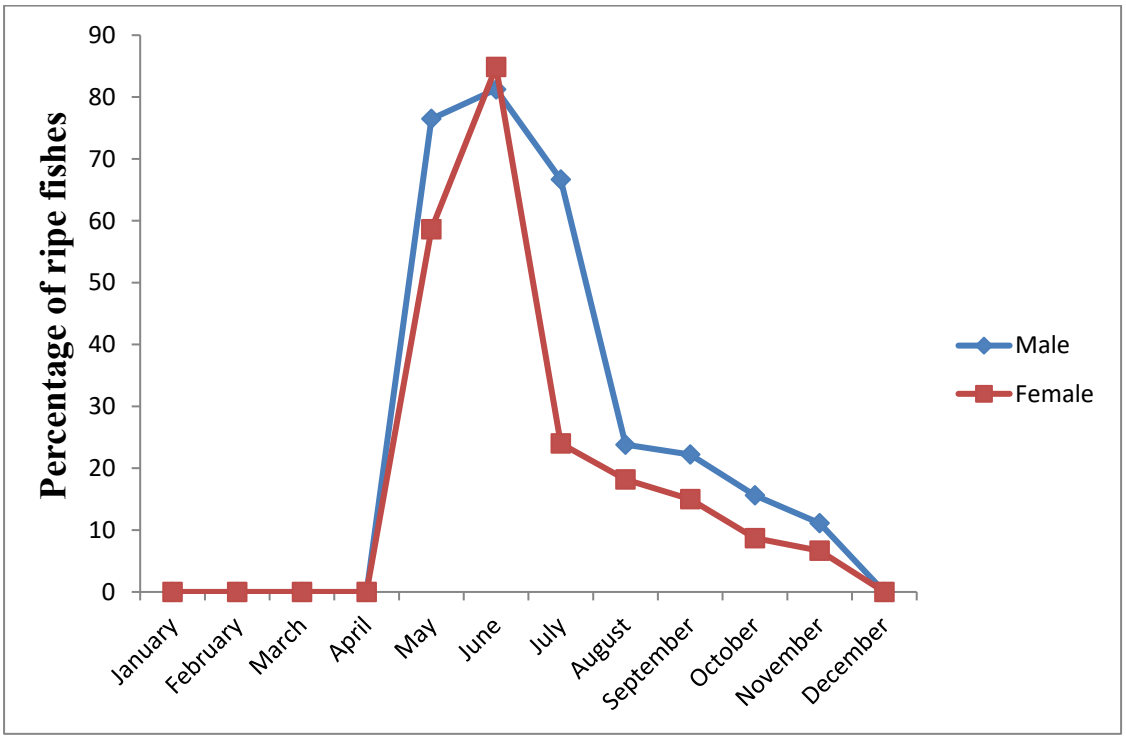


Figure 2.2, Percentage of ripe *R. dandia* among various months in the reproductive cycle

Late perinucleolus Stage: Oocytes appear as spherical or polygonal cells and become less basophilic (figure 2.6). Diameter of the cells ranges from 77.95 µm -107.85 µm (94.54 ± 9.54 µm). Nuclear diameter varies from 47.29 µm-65.99 µm (52.72 ± 5.31 µm). Nucleoli aligned along on the periphery of the nucleoplasm. Oocytes of this stage are present in all maturity stages of the ovary.

Cortical alveoli stage I: Oocyte diameter increased to a range of 131.55 µm-247.82 µm (172.53 ± 26.70 µm) in this phase of oocyte development (figure 2.7). Diameter

of the nucleus ranges from 57.43 µm-109.12 µm (79.55 ± 11.54 µm). Cortical alveoli started appearing in the cytoplasm as unstained vesicles.

Cortical alveoli stage II: Diameter of the cell ranges from 235.74 µm-318.7 µm (267.04 ± 24.66 µm) and nuclear diameter ranges from 49.44 µm-96.86 µm (82.64 ± 12.51 µm) in this phase (figure 2.8). Large number of lipid vesicles started flooding cytoplasm. Zona radiata appeared as a thin acidophilic layer around the oocyte.

Primary yolk stage: This stage is marked by the beginning of acidophilic yolk deposition (figure 2.9). Oocyte diameter ranges from 314.65 µm-392.36 µm (354.66 ± 28.07 µm). Nuclear diameter varies from 72.32 µm-113.84 µm (93.45 ± 17.18 µm). Cortical granules are still visible scattered throughout cytoplasm.

Secondary Yolk Stage: Acidophilic yolk begins filling the entire cytoplasmic area and cortical vesicles are displaced to the periphery (figure 2.10). Oocyte diameter ranges from 388.01µm-477.32 µm (436.76 ± 41.12 µm). Nuclear diameter varies from 97.36 µm -108.68 µm (104.82 ± 5.3µm).

Tertiary yolk stage: Oocytes in this phase has densely packed cytoplasm with acidophilic yolk granules (figure 2.11). Nucleoli become smaller and scattered throughout a more acidophilic nucleus. Oocyte diameter ranges from 593.36 µm-750.78 µm (683.01 ± 42.01 µm). Diameter of the nucleus varies from 94.55 µm-155.87 µm (119.31 ± 18.27 µm).

Stages of spermatocyte development

Spermatogonia: Spermatogonia are largest cell type among various stages of spermatocyte development (figure 2.12). Cytoplasm appears granular with a centrally placed nucleus. They stained weakly basophilic and noticeable in immature and

maturing testes only. Diameter of the spermatogonia ranges from 8 µm-10 µm (9.4 ± 0.84 µm).

Spermatocytes: Spermatocytes are spherical in shape and appear reduced in size compared to spermatogonia (figure 2.13). They stained strongly acidophilic and exhibited granular cytoplasm. Diameter of the spermatocytes ranges from 2.4 µm-3.2 µm (2.62 ± 0.27 µm).

Spermatids: Spermatids appear as spherical cells smaller than spermatocytes (figure 2.13). They stained strongly acidophilic. They are located towards the periphery of the testicular lobules. Diameter of the spermatocytes ranges from 1.12 µm-1.4 µm (1.24 ± 0.11 µm).

Spermatozoa: Spermatozoa were smallest of cells among various stages of spermatogenesis (figure 2.14). They stained strongly basophilic and appear as clusters towards the center of testicular lobules.

Induced breeding

Spawning response occurred to a dosage of 5000 IU (male) and 10,000 IU (female) per kg body weight of fish. Lower doses of HCG induced courtship behavior but failed in spawning response. *R. dandia* did not show spawning response to Ovaprim treatment.

Table 2.3: Response of *R. dandia* to hormone treatment for induced breeding

Treatment	Dosage per kg of body weight of fish		Response
	Male	Female	
Ovaprim	0.5ml to 2.5 ml	0.5ml to 2.5 ml	No response
HCG	1000-4000 IU	2000-8000 IU	Exhibition of courtship behavior
HCG	5000 IU	10,000 IU	Exhibition of courtship behavior followed by spawning

The following developmental stages of the *R. dandia* embryo were identified: cleavage, blastula, segmentation, pharyngula and early larva (figure 2.15).

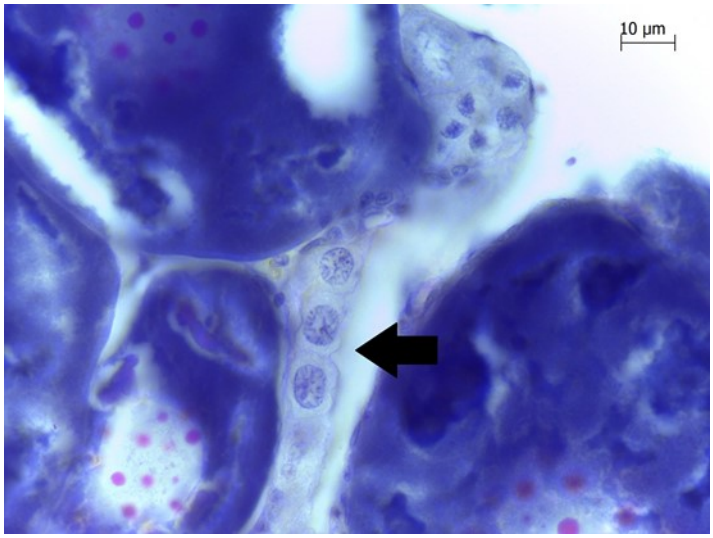


Figure: 2.3, Histological section of immature ovary of *R. dandia*, (H&E; x 1000); Oogonia cells (➡).

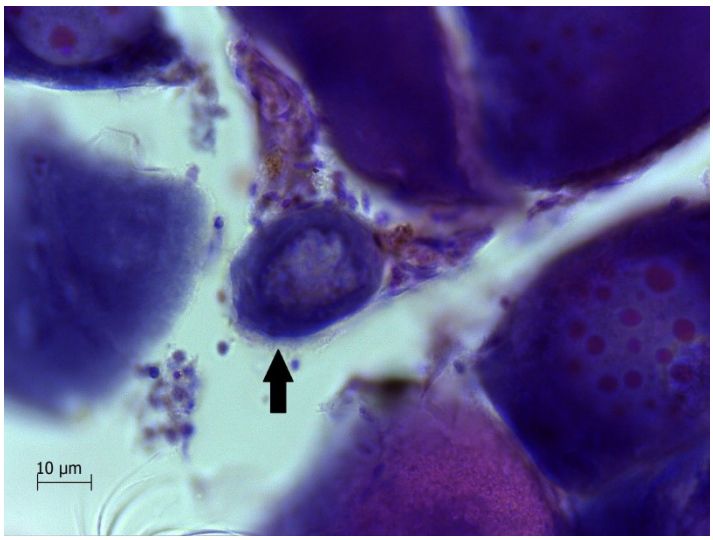


Figure: 2.4, Histological section of immature ovary of *R. dandia*, (H&E; x 1000); Oocyte in chromatin Nucleolus Stage (➡).

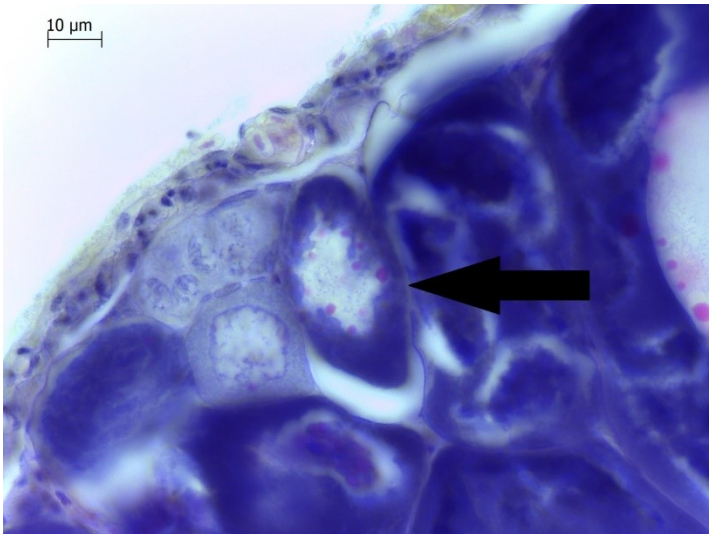


Figure: 2.5, Histological section of immature ovary of *R. dandia*, (H&E; x 1000); Oocyte in early perinucleolus Stage (➡).

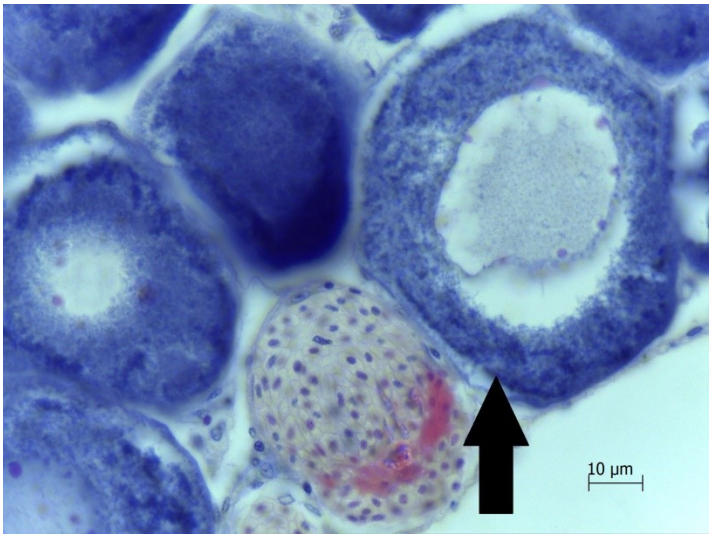


Figure: 2.6, Histological section of immature ovary of *R. dandia*, (H&E; x 1000); Oocyte in late perinucleolus Stage (➡).

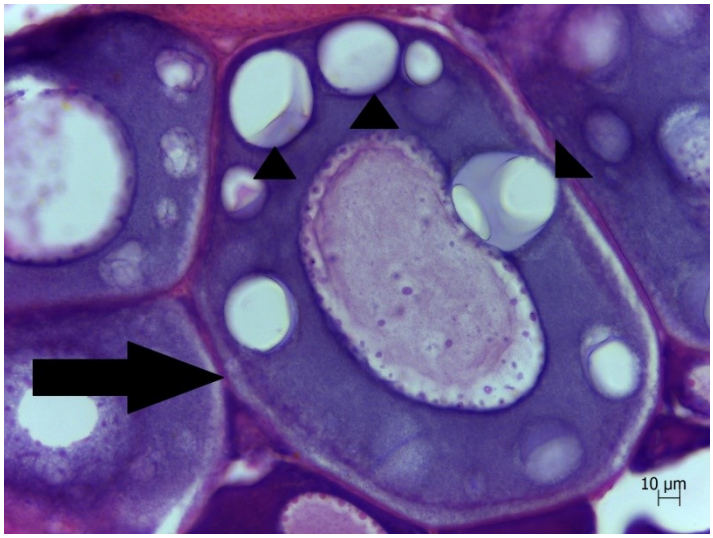


Figure: 2.7, Histological section of maturing ovary of *R. dandia*, (H&E; x 400); Oocyte in cortical alveoli stage I (➡).

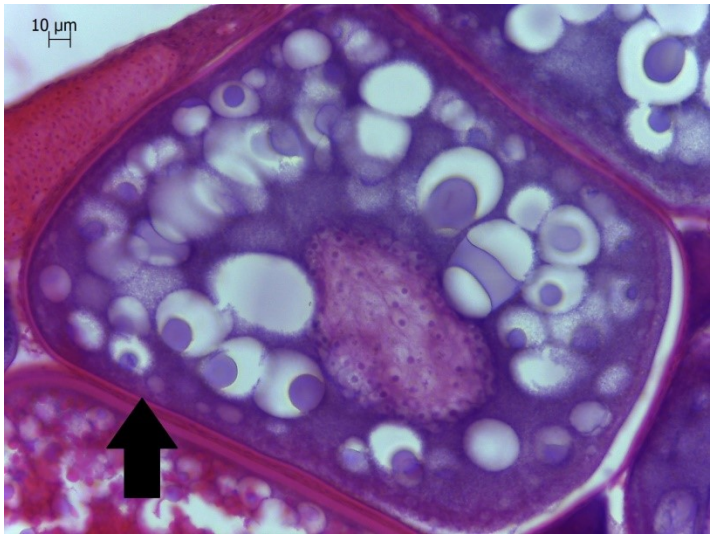


Figure: 2.8, Histological section of maturing ovary of *R. dandia*, (H&E; x 400); Oocyte in cortical alveoli stage II (➡).

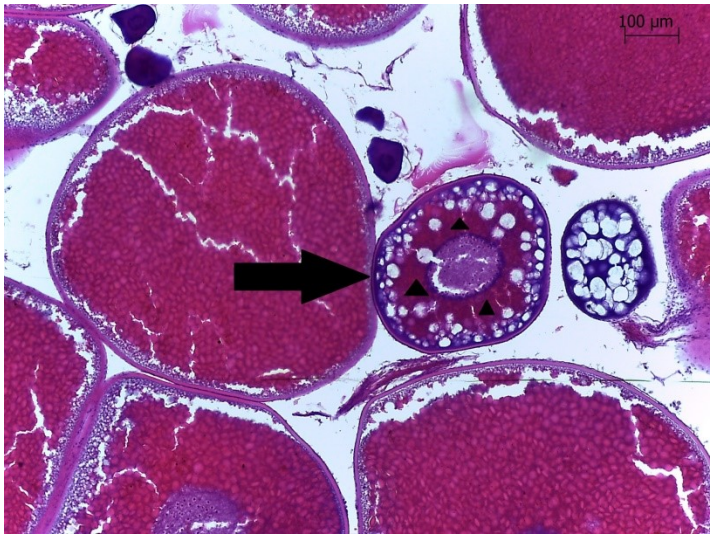


Figure: 2.9, Histological section of mature ovary of *R. dandia*, (H&E; x 100); Oocyte in primary yolk stage (➡).

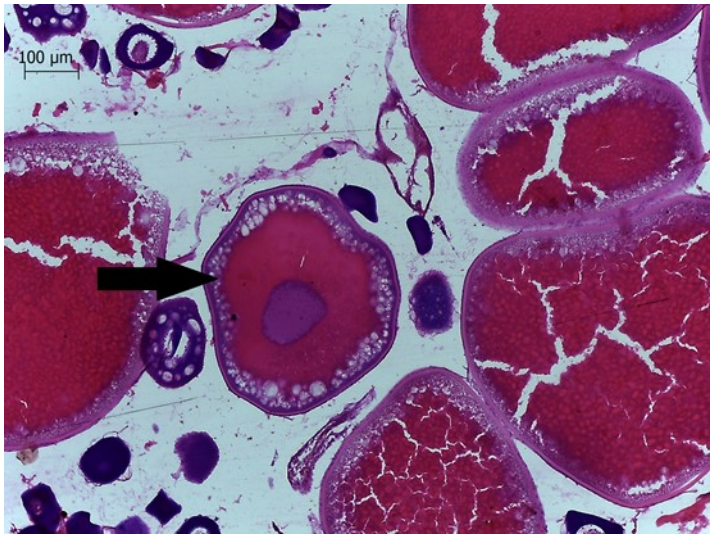


Figure: 2.10, Histological section of mature ovary of *R. dandia*, (H&E; x 100); Oocyte in secondary yolk stage (➡).

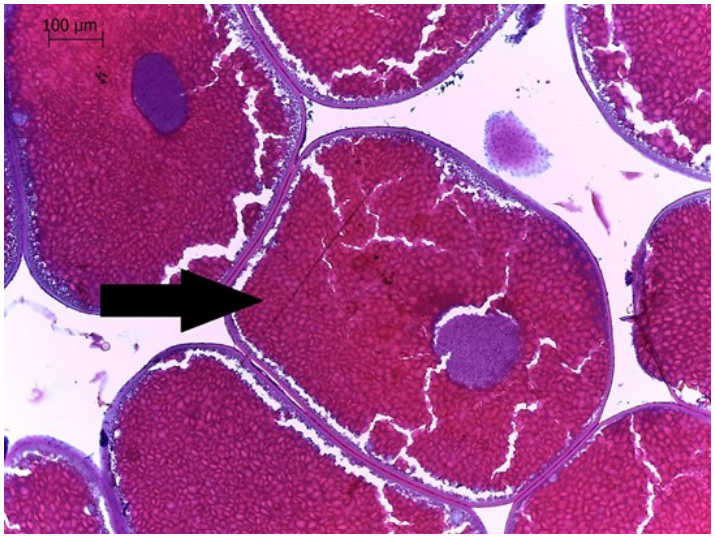


Figure: 2.11, Histological section of ripe ovary of *R. dandia*, (H&E; x 100); Oocyte in tertiary yolk stage (➡).

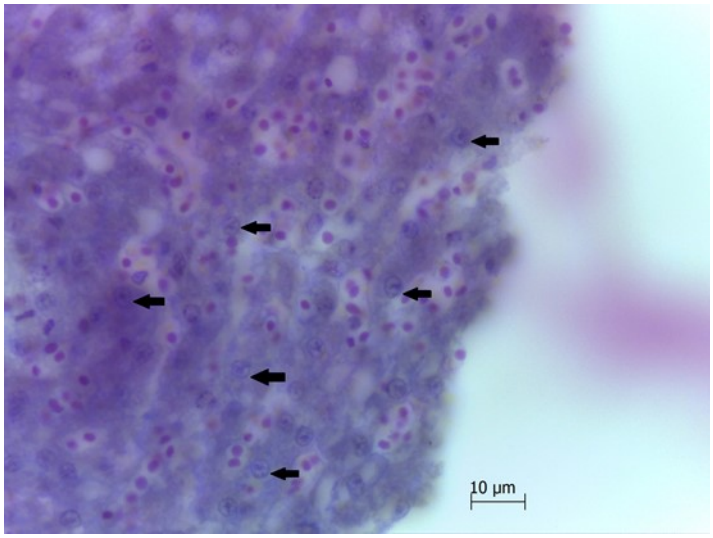


Figure: 2.12, Histological section of maturing testis of *R. dandia*, (H&E; x 100); Spermatogonia (➡).

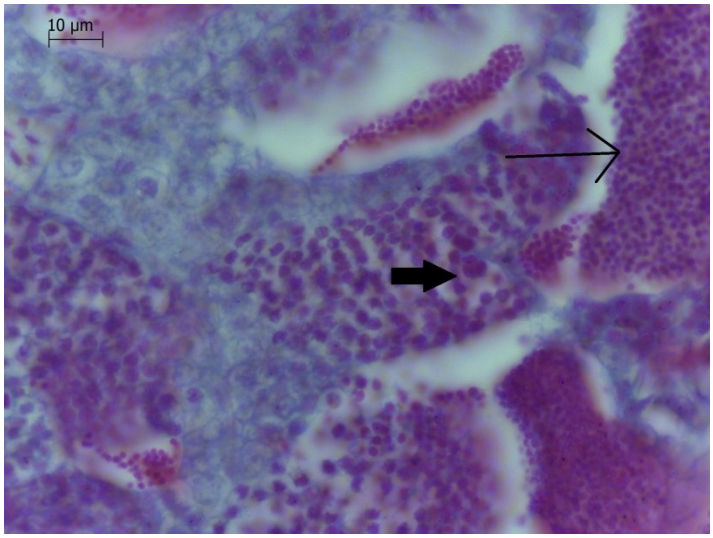


Figure: 2.13, Histological section of maturing testis of *R. dandia*, (H&E; x 100); Spermatids (→) Spermatocytes (➡).

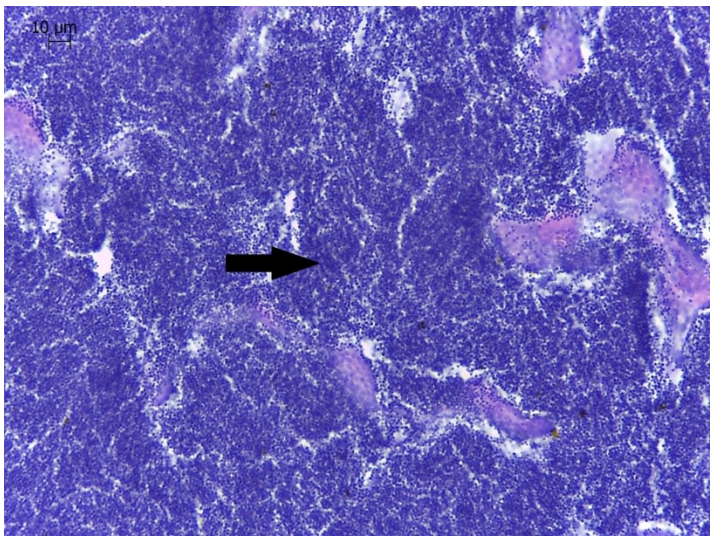
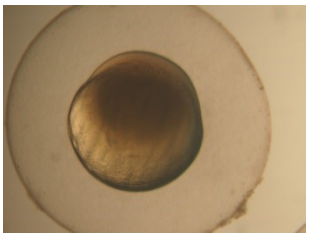
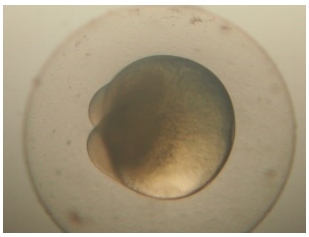


Figure: 2.14, Histological section of mature testis of *R. dandia*, (H&E; x 100); Spermatozoa (→).



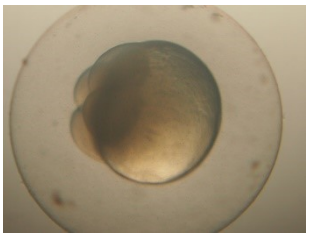
1 cell stage

(0.00 - 0.45 minutes)



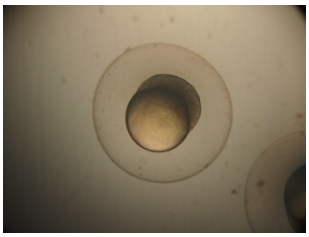
Clevage begins

(0.45 -1.30 hours)



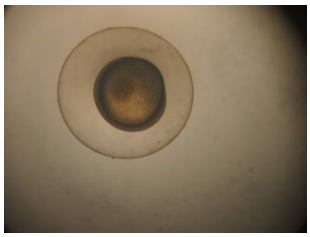
4 cell stage

(1.30 – 2.15 hours)



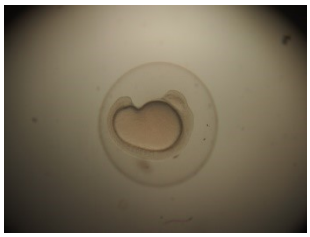
Blastula stage

(2.15 – 5.15 hours)



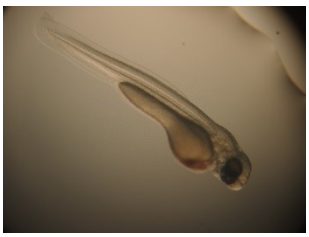
Gastrula stage

(5.15 – 10.00 hours)



Segmentation stage

(10.00 – 24.00 hours)



Pharyngula stage

(24 – 48 hours)

Hatching stage

(48 – 72 hours)

Early larval stage

(72 – 120 hours)

Figure: 2.15, Photomicrographs of developing embryos of *R. dandia.* The time line of developing embryos is given in parenthesis.

Discussion

'Leuciscus dandia' was the name Valenciennes given to a species of cyprinid fish distributed in South India, in the year 1844 (Silva *et al.,* 2010). Later the genus name was changed to *Rasbora* following Bleeker in 1859 (Liao *et al.*, 2009). However, Hamilton in 1820's was the first to use the name '*Rasbora'* in his description of *Cyprinus rasbora,* a fish known by the common name *Danikoni* or *Daniconius.* In the recent literature, however the identity of the species distributed in south India was mostly confused with this closely resembling species *viz., R. daniconius.*

Gene sequencing of cytochrome oxidase subunit 1 (CO1) of *R. dandia* and Phylogeny analysis using 658 nucleotide length of DNA sequence from the mitochondrial CO1 gene was conducted in the present study to ascertain the taxonomic status of the collected fishes. Mitochondrial CO1 sequence is known as the universal barcode sequence for identifying molecular phylogenetic relationship between animal species.

In the reconstructed phylogram (figure 2.1) the genetic identity of *R. dandia* is clear. It shows that *R. dandia* is more related to its Sri Lankan relatives, namely *R. wilpita* and *R. labiosa* and is distinct from *R. daniconius* that is distributed in the Indian mainland. Thus, the present study provided molecular evidence to the status of the species, *R. dandia.*

Maturity stages of gonads can be used as an indicator of time and duration of spawning in fishes with a regular seasonal variation in breeding cycle. When applied with care and some modifications, these categorizations are also useful in elucidating spawning seasons of fishes which produce more than one clutch annually in case of

their breeding season and only extends to one or two definite periods (Lowerre-Barbieri *et al*., 2011). In the present study, it is indicated that the spawning of *R. dandia* initiated with south west monsoon but extends up to November due to the presence of ripe stages until that month (figure 2.2). This also indicates that adult fishes may produce several batches of offspring by repeatedly gaining spawning conditions of gonads. *R. dandia* exhibit a regular pattern of annual breeding cycle with a prolonged breeding period. Abundance of rain as well as moderate climatic conditions may provide favourable breeding season throughout the year in South India.

Spawning of freshwater fishes is regulated by various environmental factors. Increasing oxygen levels of water is a major factor influencing spawning (Eggers, *et al*., 2014) and that may be the reason for migration of river fish towards inundated areas adjacent to river during monsoon. These inundated areas like paddy fields have higher oxygen levels than river as they are created by pure rain water from monsoon showers. The necessity of certain range of water temperature to successfully initiate spawning has been reported (Jonsson and Jonsson, 2009). Winters and Wheeler (1996) suggests that instead of directly influencing on spawning, water temperature influences reproductive maturation which in turn determines spawning period. Apart from other favorable physical factors of monsoon, flood conditions reduce competition for space by providing suitable breeding grounds for each species of fishes (Tedesco *et al*., 2008).

Estimation of maturity is an important tool in assessing reproductive potential and changes in biological characteristics of exploited of fish populations (Williams, 2007). Defining various stages of maturity of gonads is a common method used by

fish biologists to study pattern of reproductive cycle of fishes. It is impossible to set a common classification scheme of gonad maturity stages which are relevant to all oviparous fishes (Brown-Peterson *et al*., 2011). In the present study, five maturational stages of gonads are identified for the species *R. dandia* (Table 2.2). Classification of gonad maturational stages may be limited to five stages in tropical fishes (Selaman 1993) and too numerous distinctions of stages may be cumbersome. Gross anatomical grading of gonads suitable for routine monitoring and field studies can be used effortlessly only by reducing the number of maturity classes (Hunter and Macewicz, 2003). In a continuous breeding fish, the maturity stages can even be limited to three stages like immature, maturing and ripening (Murua *et al*., 2003; Sivakumarana *et al*., 2003).

According to Tyler and Sumpter (1996), essential pattern of oocyte development is same in all teleost fishes. Although, spermatogenesis in bony fishes resembles other vertebrates, specific differences are also present (Uribe *et al*., 2014). Spermatogenesis in fishes' proceeds through distinct stages like spermatogonia, spermatocytes, spermatids, and finally spermatozoa. Various stages like gamete development in both sexes of *R. dandia* are identified (figures 2.3. to 2.14) in the present study shows that the development stages are the same as in other teleosts. This aids in the assessment of progress of gonad recrudescence in fishes under toxicant stress as well as in control fishes. Alternation in diversity and abundance of various stages of gametes gave hints of histopathological changes in the presence of toxicants. Different phases of early development of *R. dandia* were identified in the present study based on morphological peculiarities. Fish embryos from different clutches may exhibit asynchrony and a morphological criterion is more suitable for staging embryos than a chronological distinction (Kimmel et al. 1995). The stages of developing

embryos of *R. dandia* (figure 2.14) are also same as in other related species of teleost fishes (Kimmel *et al*., 1995; Dey *et al*., 2014).

Induced breeding of *R. dandia* under laboratory conditions were successful with the administration of HCG hormone (Table 2.3). The ability of oocytes to respond to steroidal maturation-inducing hormone or the maturational competence in oocytes of fishes might have been enhanced by gonadotropins (Kagawa *et al.,* 1994; Yoshizaki *et al*., 2001). Patiño and Thomas, (1990) investigated the mechanism of maturational competence in Atlantic croaker oocytes induced gonadotropin. A steroid independent priming phase, followed by germinal vesicle breakdown facilitated by maturation-inducing steroid are the two distinct phases of final oocyte maturation occurred under the influence of gonadotropin in croaker oocytes. HCG induces steroid production in vitellogenic follicles as well as germinal vesicle breakdown in fish oocytes (Degani and Boker, 1992; Srivastava and Van Der Kraak, 1994; Chourasia and Joy 2008). HCG treatment blocks resorption of milt and induces spermiation in male fishes (Park *et al*., 2002; Asturiano *et al*., 2006).

Eurasian perch, *Perca fluviatilis* was induced to spawn administering 5700 IU and 2850 IU per kg HCG in female and male fishes respectively (Kucharczyk *et al.,* 1996). According to García-Alonso and Vizziano (2004), 300 IU HCG per kg of body weight is the dose stimulating oocyte maturation and natural ovarian dynamics rapidly in white croaker, *Micropogonias furnieri*. Haniffa and Sridhar (2002) observed successful spawning in *C. punctatus* with 2000 and 3000 IU per kg body mass of HCG and *Heteropneustes fossilis* with 1000, 2000, and 3000 IU per kg body mass of HCG. *R. dandia* spawned successfully at a much higher dose of 5000 IU (in male) and 10,000 IU (in female) per kg body weight of fish.

Tropical marine and freshwater fishes are commonly induced to spawn with a lower priming dose followed by higher resolving dose with an interval of 3-24 hours depending on the species (g and Lam, 1996). Cacot *et al*. (2002) induced spawning of P*angasius bocourti* by successive HCG treatments. Lower doses of HCG were given to induce development of ovarian follicles as a preparatory treatment followed by higher dose which led to ovulation. Repeated handling for successive injections will be stressful especially in a small sized fish like *R. dandia*. Campbell *et al*. (1992) found out that when rainbow trout was exposed to stress prior to spawning, gamete quality is reduced significantly. Capture and handling lead to stress in fishes leading to variation in the levels of reproductive hormones along with the induction of stress hormone, cortisol (Harvey and Carolsfeld, 1993). Thus, in the present study induced breeding is achieved by administering a single dose of HCG, avoiding preparatory doses to ensure minimum stress to the breeding fishes.

Being an important food source, fishes are a class of organisms which has profound importance to human beings. The economic importance of fishes provides powerful motivation for large scale of studies building a rich database on dynamics of fish populations (Jakobsen *et al*., 2009). As reproductive characteristics play an important role in determining a population's resilience to exploitation, a core theme of scientific advice to fishery management must be reproductive biology (Morgan, 2008). Reproduction is directly linked to the productivity and abundance of the fish stocks. Stock assessment models helps in the rational management of fisheries by assessing the information like spawning potential and recruitment (Pope, 2016). Stock assessment models require knowledge on growth and reproduction as input data (Cherif *et al*., 2007). Availability of wide databases on reproductive parameters combined with data on corresponding abiotic factors facilitates the investigations on

the relationship between environmental variation and reproductive potential (Murua *et al*., 2003). Harvest recommendations provided in fisheries are based on the spawning potential of fish populations, assessed by information on reproductive condition collected by sampling of commercial catches and biological surveys (Williams, 2007). Exploitation of fish resources during breeding season impairs future fish recruitment and dwindle valuable fish stocks. Ban on fish catchment are often implemented by government bodies during critical periods related to fish recruitment. Extensive research on reproductive biology enables precise implementation of legislatures regarding sustained utilisation of fisheries wealth.

CHAPTER 3

LETHAL TOXICITY STUDY AND BIOACCUMULATION OF HEAVY METALS IN THE REPRODCUTIVE ORGANS

Introduction

Toxicological investigations begin with the evaluation of acute toxicity of the proposed toxicant. While studying the impact of toxicants in the environment of an organism like xenobiotics in the water or poisonous vapours, the concentration in the medium (LC_{50}) is considered. Median lethal concentration provides an index of acute toxicity. According to Aldenberg and Jaworska (2000) while selecting the method, practicality deserves much attention as the statistical and theoretical considerations and hence, methods that calculate both the LC_{50} and its 95 percent confidence limits should be accepted. The most commonly used methods for calculating median lethal concentration are integrated normal (probit) models. The relationship between mean mortality and the concentration of toxicant are depicted by these models (Landrum *et al.,* 2012; Eugene *et al*., 2017). A range of computational and graphical methods can be applied to obtain a median lethal concentration from mortality data on different concentrations derived from an acute mortality test.

The LC_{50} test is applied to establish the sensitivity of various species to toxicant pollutants and the outcomes are applied to predict potential impact on human health and detrimental effect on wild populations (Clingerman, 1990). The sensitivity of test organisms to varying concentrations of a toxicant are scrutinized in environmental toxicological studies and survival is a regular dichotomous response evaluated in them. A comparison of the potency of a toxicant between different populations can be made with statistical analysis of differences in LC_{50} values (Wheeler *et al.,* 2006). Data on lethal concentration of various pollutants on native species facilitate designing further toxicology investigations. Extensive toxicity studies provide information to identify sensitive group of organisms. In addition,

toxicity data provide substantial background information on setting water quality criteria on various ecosystems.

Rajan and Banerjee, (1991) evaluated 96 hour LC_{50} on catfish, *H. fossilis* with mercuric chloride as toxicant and obtained the value of 0.27 ppm using 24 hour renewal bioassay system. Vieira *et al.* (2009) exposed the estuarine fish, *Pomatoschistus microps* to varying concentrations of mercuric chloride. 228.60 ppb, 130.30 ppb, 71.03 ppb and 61.89 ppb were the LC_{50} values obtained for 24 hours, 48 hours, 72 hours and 96 hours respectively. The acute toxicity of mercury to black fish, *Capoeta fusca* was investigated by Mansouri *et al.* (2012) using mercuric sulphate. The LC_{50} values were found to be 0.24 mg L^{-1} for 96 hours of exposure.

LC_{50} values of Pike, *Esox lucius* were 78 ppb and 92 ppb on 96 hours and 24 hours respectively with mercuric chloride (Rahimibashar and Alipoor, 2012). Caspian roach, *Rutilus caspicus* has a median lethal concentration of 330 ppb of mercury for 96 hours (Hoseini and Nodeh, 2012). Static bioassays were conducted on Indian major carp, *Labeo rohita* to find median lethal concentration of mercury using mercuric chloride. 810.88 ppb, 710.90 ppb and 651.76 ppb were the LC_{50} values obtained for 24 hours, 48 hours and 72 hours respectively (Ghosh and Mandal, 2013). Banavathu *et al.,* (2016) obtained 96 hour LC_{50} value of 0.25 ppm for *L. rohita,* under the exposure of mercuric chloride. Snarski and Olson (1982) exposed fathead minnows, *Pimephales promelas* to different concentrations of mercuric chloride in water to determine its acute toxicity. 168 ppb, 112 ppb, 84 ppb, and 74 ppb of mercury for 4, 5, 6, and 7 days respectively were the mean LC_{50}values obtained. Hedayati *et al.,* (2013) exposed common carp, *Cyprinus carpio* to mercuric chloride and 96 hour LC_{50} obtained was 0.93 ppm.

Ullah *et al.,* (2016) exposed *L. rohita* to cadmium sulphate and 96 hour LC_{50} was found to be 24 ppm. Thophon *et al.,* (2003) exposed *Lates calcarifer* to cadmium chloride and the 96 hour LC_{50} value obtained was 20 ppm. Dutta and Kaviraj (2001) found 96 hour LC_{50} of cadmium as 89.5 ppm in *L. rohita.* Sobha *et al.,* (2007) exposed *Catla catla* to cadmium chloride of varying concentrations from 1 ppm to 8 ppm. 96 hour LC_{50} of cadmium was found to be 4.53 ppm. 96 hour LC_{50} was determined to be 50.41 ppm for *H. fossilis* exposed to varying concentrations of cadmium chloride (Singh *et al.,* 2010). Catfish, *Pangasius hypophthalmus* and cyprinid, *C. carpio* have 96 hour LC_{50} values of 64.89 ppm and 84.8 ppm respectively for cadmium chloride (Abedi *et al.,* 2012).

Bioaccumulation is a process in which a chemical substance is absorbed in an organism by all routes of exposure as occurs in the natural environment, through dietary and ambient environment sources (Arnot and Gobas 2006). According to International Union of Pure and Applied Chemistry (IUPAC) recommendation (1993), bioaccumulation is the "progressive increase in the amount of a substance in an organism or part of an organism which occurs because the rate of intake exceeds the organism's ability to remove the substance from the body" Along with the availability of pollutants, biological, chemical and environmental factors control the rate of bioaccumulation. The ability to store or excrete pollutant by the organism is most important among them (Walker, 2009). Polychlorinated biphenyls, dioxins, organochlorine pesticides and heavy metals are the major bioaccumulating pollutants in various ecosystems (Albanis *et al.,* 1996; Streit,1998; Wu *et al.,* 2001; van der Oost *et al.,* 2003; Weisbrod *et al*., 2009 a,b; Schäfer *et al*., 2015).

Heavy metals enter aquatic ecosystems from anthropogenic sources like domestic sewage, industrial effluents, mining related activities and fossil fuel combustion (Chmielewski, 2002; Armah *et al.,* 2010; Li and Tse, 2015). Natural phenomenon like volcanic eruptions and weathering also result in heavy metal pollution in the environment (Starr *et al.,* 2003 and Deolsch *et al.,* 2006). As heavy metals cannot be degraded, they always present and redistributed among various components in aquatic ecosystems (Streit 1998; Linnik and Zubenko, 2000). Property of bioaccumulation makes the hazard of heavy metal pollution more vicious in an ecosystem. As each trophic level is succeeded, organisms get exposed to heavy metals in a more concentrated level than those in the previous one.

Eagles-Smith *et al.* (2009) investigated mercury accumulation in water bird species of various guilds in San Francisco Bay, California. Highest mercury levels were found in fish eating Forster's terns, *Sterna forsteri* and Caspian terns, *Hydroprogne caspia.* Concentrations were elevated in black-necked stilts, *Himantopus mexicanus* which consume primarily, invertebrates. Foraging habitat, trophic position and exposure time influenced mercury levels in bird species. Breeding water birds were at the increased risk of mercury influenced reproductive impairment. Bastos *et al.,* (2007) studied the annual flooding and mercury bioaccumulation in fishes of environmentally impacted Amazonian River Madeira. Mercury concentrations of predatory species were much higher than that of the detritivorous species.

Arantes *et al.,* (2016) collected catfish, *Pseudoplatystoma corruscans* from two sites on the Paraopeba River of Brazil and investigated the concentrations of cadmium, zinc, mercury, lead and chromium in muscle, liver and spleen tissues.

Visceral organs accumulated high concentration of heavy metals than muscle suggesting viscera unfit for human consumption. Correlations between fish and heavy metal levels were not found significant in the study.

Mercury concentrations were estimated in summer flounder, *Paralichthys dentatus* from New Jersey coastal waters and compared with the polychlorinated biphenyl concentration from a previous study. Polychlorinated biphenyl concentration was 43% greater in males than in females, whereas as in the case of mercury 2% elevation is found in females than in males. Results indicated that sex difference significantly influenced the type of contaminant in fishes (Madenjian *et al.,* 2016).

Koffi *et al.* (2014) investigated the concentration of cadmium, lead and mercury in different tissues of various tuna species collected from the coastal zone of Cote d'Ivoire. Highest concentrations of the three heavy metals were observed in the gills and the liver while gonads were the least contaminated. Kasper *et al.,* (2009) estimated the levels of organic and inorganic mercury in different fishes from various trophic levels of tropical reservoir in southeastern Brazil. Gonads accumulated the lowest amount of total mercury and most of it was in organic form.

Nanda, (2014) exposed *Anabas testudineus* to paper mill effluent with the concentration of ≤ 0.2 ppm of different heavy metals. Interestingly, beside the gills and liver, ovary was found to be a target organ for metal accumulation with highest maximum accumulation factor. Kumar *et al.,* (2015) evaluated bioaccumulation of zinc in fresh water fish, *C. punctatus* by chronically exposing the fishes to zinc sulphate. The level of zinc accumulation in the gonads of fish was lower than the organs like liver, kidney, gill and muscle. Verma and Srivastava, (2008) observed that the zinc accumulation is linearly proportional to the dose and duration of exposure in

C. punctatus. Mohanambal and Puvaneswari, (2013) evaluated the accumulation of lead in various tissues of *C. catla* exposed to a sublethal concentrations of lead for a period of 120 days. Gonads showed minimum accumulation of lead compared to liver, gills and kidneys. Although liver, kidneys and gills are the major target organs of heavy metal bioaccumulation, gonads are also able to accumulate a significant amount of heavy metals. In order to get an understanding of the nature of bioaccumulation of mercury and cadmium in the gonads of sexually mature *R. dandia*, test fishes were exposed to mercury and cadmium and accumulation levels in the gonads of the fish were investigated.

Materials and methods

Test organism

Acclimatized fishes were maintained as described in chapter 2, and fishes with 5.5.to 7.5 cm standard length and 2.5 to 5.5 gm weight were selected. 10 fishes were kept in each glass aquarium.

Lethal toxicity study

Stock solutions of mercuric chloride (Loba Chemie, India, extra pure grade) and cadmium chloride (Merck India, analytical grade) were prepared in double distilled water and were used as the source for mercury and cadmium respectively. The stock solution was kept at 4º C and all toxicity test concentrations were prepared from the stock solution. A range of test concentration of mercury and cadmium was determined based on the results of a preliminary study. Calculated volume of mercuric chloride was added carefully to the water in the aquarium so as to get varying doses of mercury viz., 80 ppb, 100 ppb, 120 ppb, 140 ppb and 160 ppb. Required volume of stock solution was added to the water in the aquaria so as to get

doses of cadmium viz., 10 ppm, 12 ppm, 14 ppm 16 ppm and 18 ppm. Water in the aquaria was stirred briskly so as to distribute the stock solution uniformly in the water. The physico-chemical conditions of the water in the aquaria and other conditions of the water in the aquaria were maintained as described in chapter 2. Water of the aquaria was renewed and calculated volume of the stock solution was added every 24 hours. Ten test organisms were exposed to each of the metal concentration of the aquaria. A control group was maintained for the study of each metal without adding any metal to the water. Number of mortalities in each aquarium was recorded on every 12 hours for an experimental period of 96 hours. The dead organisms were removed from the aquaria regularly. The experiment was carried out under a completely randomized manner with one replication for each metal concentration.

Bioaccumulation study

R. *dandia* fishes were maintained in the laboratory as described in chapter 2 of the thesis. Thirty male fishes were selected and divided into three equal groups. Two experimental fishes were exposed to 0.01 ppm of mercury and 1 ppm of cadmium respectively for thirty days. The third group was served as control. Test solutions were aerated and renewed at every 24 hours. Five fishes each were selected at random, anaesthetized and sacrificed to dissect the testis at ten days' time interval for 30 days. The experiment was repeated for three times. In a similar manner female *R*. *dandia* fishes were maintained and exposed to 0.01 ppm mercury and 1.00 ppm of cadmium for thirty days. Five female fishes were selected at random and ovaries were dissected out carefully. The experiment was repeated for three times. The collected tissues were further processed for mercury and cadmium analysis.

Heavy metals were estimated according to slightly modified method of Bahorom and Ishak (2015). Briefly, 0.50 g wet gonads were weighed accurately and transferred into microwave digestion tubes. Self digestion was allowed for 15 minutes with 0.50 ml of concentrated HNO_3 (extra pure), 0.5 ml of concentrated HCl (extra pure), and 1 ml of H_2O_2. After the self digestion, the caps were tightened and kept for digestion in a microwave digestion system (MDS) (Mars Xpress, CEM Corporation, USA) for 20 minutes at a temperature of 180ºC. After digestion, the contents from MDS digestion tubes were quantitively transferred to 50 ml tube and made upto 50 ml using extra pure water. Samples were analysed using an inductively coupled plasma mass spectrometer (ICP-MS) (model 7700, Agilent, USA). Samples were introduced into the nebulizer of ICP-MS for the analysis using a peristaltic pump and the results were expressed as µg/gm of tissue weight.

To arrive at the concentrations of heavy metals in gonads, stock solutions of mercuric chloride (Loba chemie, India, extra pure grade) and cadmium chloride (Merck India, analytical grade) at a concentration of 1 µg/g were prepared. Working standards were prepared from stock solution by appropriate dilution so that all the standards fall within the linear range of the element of interest. Mixed solutions of mercury and cadmium were prepared in the following concentrations and used as reference: 5 µg/kg, 50 µg/kg, 100 µg/kg, 200 µg/kg and 250 µg/kg. Water samples were drawn from each aquarium during 10 days' time interval and analyzed for the mercury and cadmium concentrations as described above.

The bioaccumulation factor (Bf) is calculated using the equation log (Cb/Cw) where Cb is the level of heavy metal in the gonadal tissue and Cw is the level of heavy metal in the medium (Arnot and Gobas, 2016).

Statistical analysis

SPSS software 16.0 was used for carrying out statistical analysis. Probit analysis was used to calculate LC_{50} value and its 95% confidence limits (Finney, 1971). Significance of bioaccumulation was analysed using one way analysis of variance (ANOVA) along with Tukey's post-hoc test.

Results

Fish mortality was found to be significantly increased with increasing concentration and exposure period of mercury and cadmium. No mortality was observed in the control group (Tables 3.1 and 3.2). 96 hours LC_{50} concentration of mercury and cadmium was found to be 133.3 ppb and 16.91ppm respectively (table 3.3 and figures 3.1 and 3.2).

In bioaccumulation study cadmium and mercury were undetected in control samples. Mercury was found to be accumulated in the gonads of test fishes exposed to mercury. By 10 days of mercury exposure, both testes and ovaries accumulated mercury with ovaries showing slightly higher accumulation than testes. Both testes and ovaries showed similar level of mercury accumulation at 20 days of exposure period. By 30 days of exposure, testes did not show further increase in accumulation of mercury while ovaries showed an increase in mercury accumulation (figure 3.3). Test fishes exposed to cadmium showed accumulation of cadmium in the gonads. The results obtained indicated increased accumulation as exposure period progresses. Both testes and ovaries showed similar pattern of cadmium accumulation (figure 3.4). The Bf, presented in table 3.4 shows that, Bf varied slightly in 10 days, 20 days and 30 days for mercury in testis and ovary of the fish. In the case of cadmium, the Bf is higher in testis (1.60 to 2.51) than in ovary (0.19 to 0.42).

Table 3.1: Showing mortality of *R. dandia* in every 12 hours by the exposure of mercuric chloride

Experimental Concentration(ppb)	12	24	36	48	60	72	84	96	Total
80	1	Nil	Nil	Nil	Nil	Nil	Nil	Nil	1
100	1	Nil	Nil	Nil	Nil	Nil	Nil	1	2
120	1	Nil	Nil	Nil	Nil	Nil	1	1	3
140	1	1	Nil	Nil	Nil	Nil	Nil	2	4
160	1	1	Nil	Nil	Nil	Nil	1	6	9
control	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil

Table 3.2: Showing mortality of *R. dandia* in every 12 hours by the exposure of cadmium chloride

Experimental Concentration(ppm)	12	24	36	48	60	72	84	96	Total
10 ppm	Nil	Nil	Nil	Nil	Nil	Nil	1	Nil	1
12 ppm	Nil	Nil	Nil	Nil	Nil	1	1	Nil	2
14 ppm	Nil	Nil	1	Nil	Nil	Nil	1	1	3
16 ppm	Nil	1	Nil	Nil	1	Nil	2	Nil	4
18 ppm	1	Nil	Nil	1	Nil	2	2	Nil	6
control	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil

Table 3.3: Showing LC_{50} value of *R. dandia* for mercury and cadmium

Toxicant	LC_{50}	Upper confidence limit	Lower confidence limit
Mercury	133.3 ppb	162.04	116.45
Cadmium	16.91ppm	34.08	14.562

Table 3.4. Showing bioaccumulation factor (Bf) of *R.dandia* for thirty days when they are exposed to 0.1ppm of mercury and 1.00 ppm of cadmium.

No. of days	Mercury		Cadmium	
	Testis	Ovary	Testis	Ovary
10	2.11	2.16	1.60	0.19
20	2.32	2.32	2.25	0.30
30	2.33	2.33	2.51	0.42

The bioaccumulation factor (Bf) is calculated using the equation log (Cb/Cw) where Cb is the level of heavy metal in the gonadal tissue and Cw is the level of heavy metal in the medium (Arnot and Gobas, 2016).

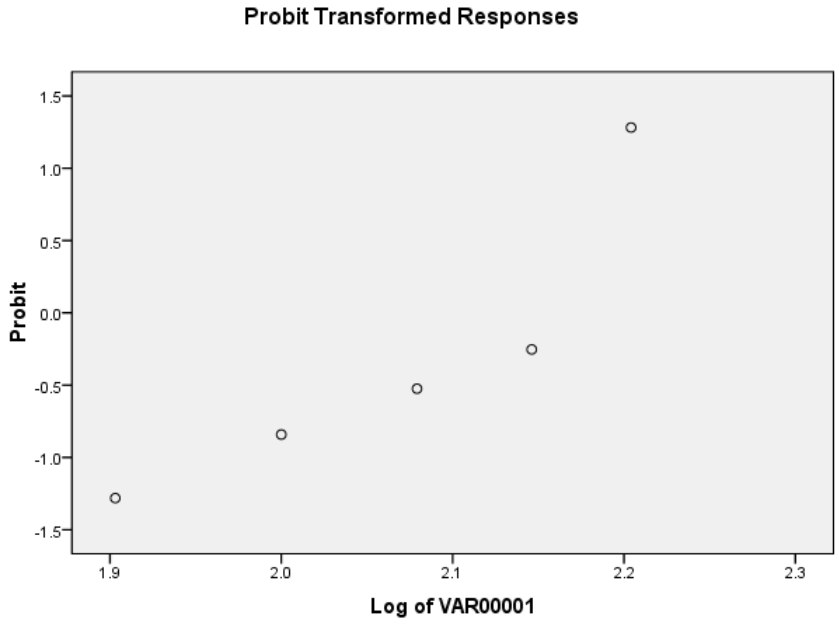


Figure: 3. 1, Probit graph of 96 hours LC_{50} of *R. dandia* for mercury.

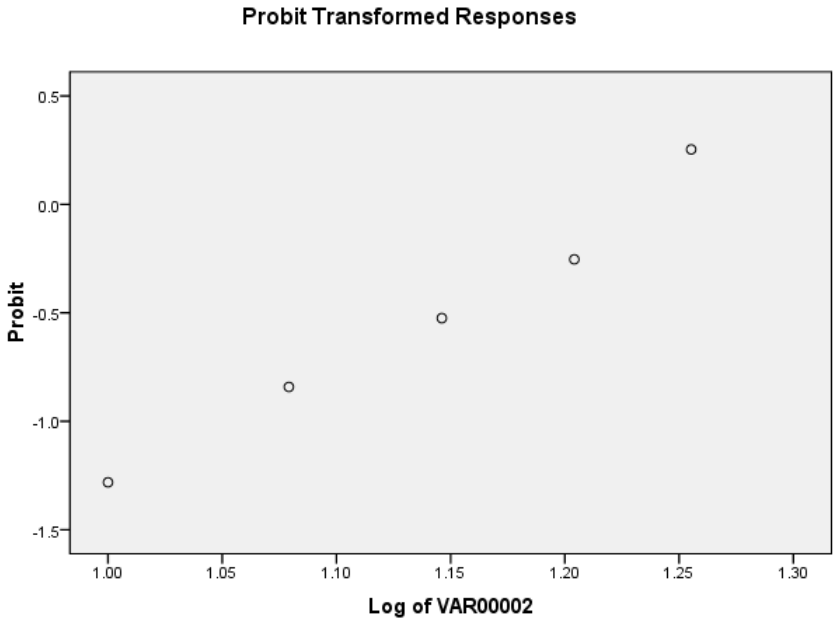


Figure: 3. 2, Probit graph of 96 hours LC_{50} *R. dandia* for cadmium.

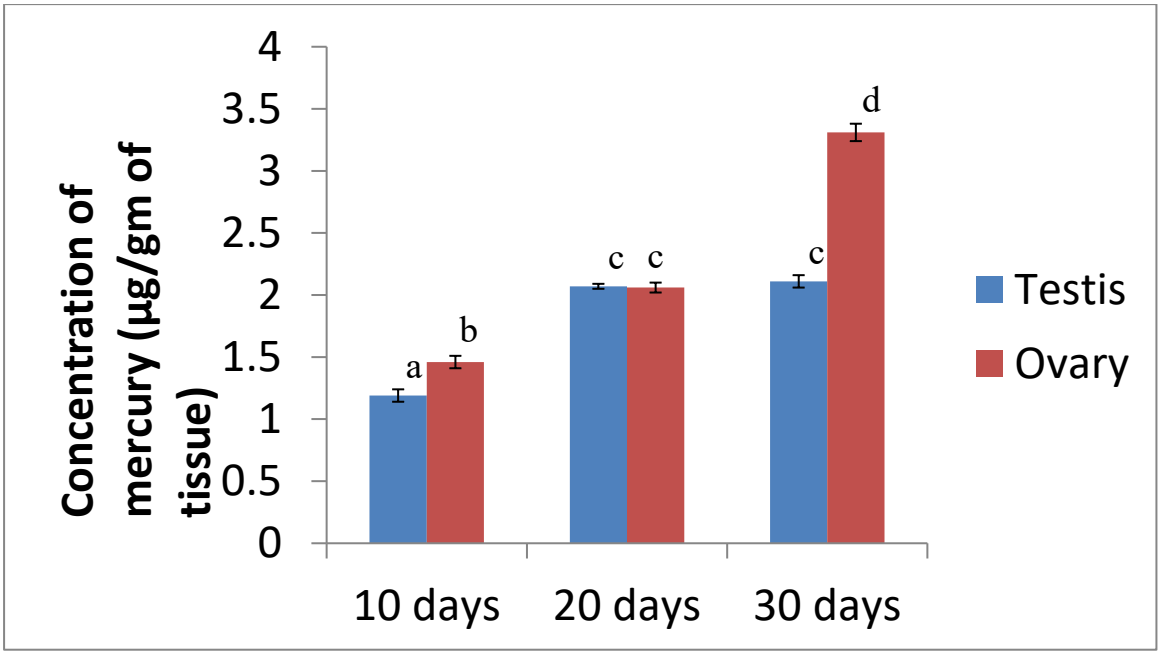


Figure: 3. 3, Accumulation of mercury in the gonads of *R. dandia* (Mean ± S.D). Results are the mean of three replicates of each group. Different superscript letters indicate significant variation between exposure groups ($p<0.05$; One way-ANOVA, Tukey's post-hoc test).

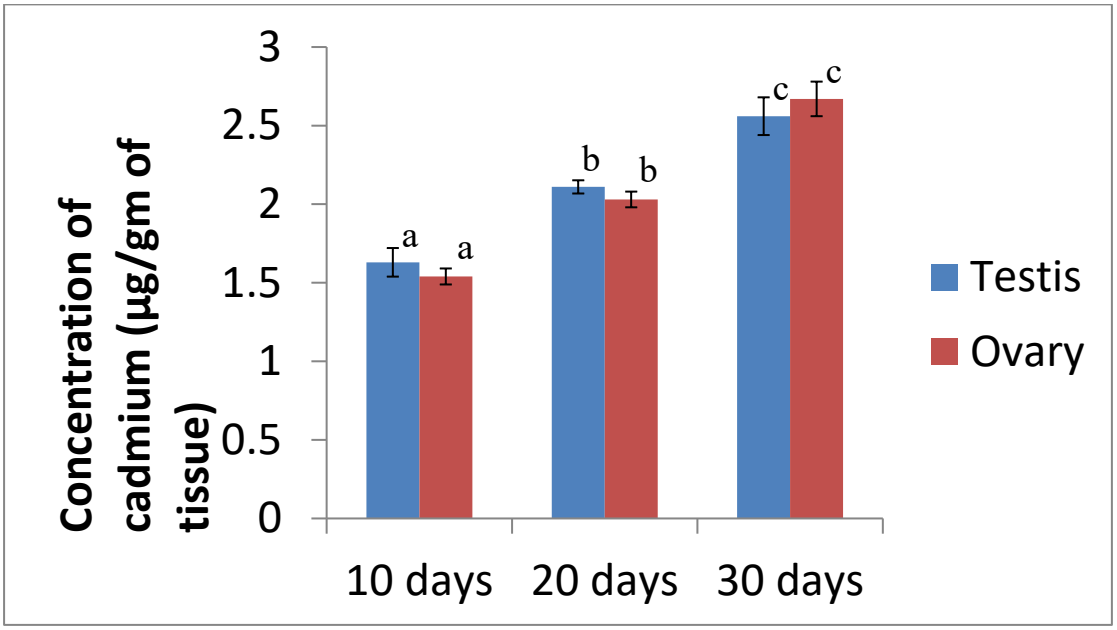


Figure: 3. 4, Accumulation of cadmium in the gonads of *R. dandia* (Mean ± S.D). Results are the mean of three replicates of each group. Different superscript letters indicate significant variation between exposure groups ((p<0.05; One way-ANOVA, Tukey's post-hoc test).

Discussion

Sensitivity of various fishes to the toxicant effects of mercury varies widely among different species (Rajan and Banerjee, 1991; Vieira *et al.,* 2009; Mansouri *et al.,* 2012; Ghosh and Mandal, 2013). In the present study *R. dandia* was exposed to mercuric chloride and 96 hours LC_{50} concentration found to be 133.3 ppb of mercury which varies from other cyprinid fishes (Table 3.3). Hoseini and Nodeh, (2012) determined acute toxicity of mercury on Caspian roach *R. caspicus* and 96 hour LC_{50} was found to be 330 ppb of mercury. 96 hour LC_{50} on pike, *E. lucius* were 78 ppb with mercuric chloride (Rahimibashar and Alipoor, 2012). Common carp, *C. carpio*, were exposed to mercuric chloride 96 hour LC_{50} was found to be 0.93 ppm (Hedayati *et al.,* 2013). Heterogeneous nature of response to the level of mercury even among related species implies the necessity of wide range of acute toxicity data to evaluate the impact of different levels of toxicant contamination on aquatic ecosystems.

Singh *et al.,* (2010) exposed *H. fossilis* to varying concentrations of cadmium chloride and 96 hour $LC_{50}$was determined to be 50.41 ppm. Abedi *et al.,* (2012) exposed catfish, *P. hypophthalmus* and cyprinid, *C. carpio* to cadmium chloride and compared median lethal concentration. 96 hour $LC_{50}$was found to be 64.89 ppm and 84.8 ppm for *P. hypophthalmus* and *C. carpio* respectively. 96 hour LC_{50} of *L. rohita* for cadmium by static bioassay was found out to be 89.5 ppm (Dutta and Kaviraj, 2001). 96 hour LC_{50} value of 16.91ppm of cadmium for *R. dandia* obtained in the present study (table 3.3) is lower considering these previous studies which may be attributed to the smaller size of the fish. 96 hour LC_{50} value of 9.68 ppm of cadmium was calculated for zebra fish, *D. rerio* which is a small cyprinid fish (Al-sawafi *et al.,* 2017) and is comparable to the dose obtained in the present study.

Inorganic mercury in the water enters fishes with gills being the major route of uptake (Simon and Boudou, 2001; Bradley *et al*., 2017). Acute exposure of mercuric chloride induces microscopic lesions like lamellar fusion and lamellar epithelial cell apoptosis contributing to low oxygen levels, severe enough to cause mortality (Monteiro *et al*., 2008). Decreased oxygen consumption is observed in the liver, gills and muscle of *C. carpio* following one day exposure to 0.1 ppm of mercury (Radhakrishnaiah *et al.,* 1993). Gills have major osmoregulatory functions in fishes which may be altered by mercuric chloride. Permeability characteristics of water in the gills altered and inhibited $Na^+ - K^+$ ATPase activity at lethal concentrations of mercury (Poopal *et al.,* 2013).

Inorganic mercury being a neurotoxin caused behavioural changes like surfacing, erratic movement, loss of balance and inactivity under acute exposures in fishes (Banavathu *et al.,* 2016; Pereira *et al*., 2016). Mercury disrupts chemoreception

in fish by affecting taste and olfactory receptors (Baatrup 1991; Døving 1991; Klaprat et al., 1992). Serum ions are found to be increased in catfish, *Clarias lazera* under mercury exposure (Hilmy *et al.,* 1987). Anaerobic and aerobic metabolism is disturbed in fishes exposed to mercury. Rosy barb, *Puntius conchonius* exhibited suppressed alkaline phosphatase activity in gills, liver, kidneys and gut where as it elevated in the gonads under short term exposure to high mercury concentration. Aspartate aminotransferase and alanine aminotransferase were also inhibited in various tissues. Cardiac and skeletal muscles showed elevated lactic dehydrogenase activity whereas considerable lowering of acetylcholinesterase occurred in liver, gills and brain (Gill *et al.,* 1990). Succinic dehydrogenase is suppressed while lactate dehydrogenase is elevated in muscle, liver and gill of *C. carpio* under mercury exposure (Radhakrishnaiah *et al.,* 1993).

Routes of cadmium uptake in fishes are gills, intestine and skin. Stomach as well as various sections of intestine participates in cadmium uptake. Skin absorbs relatively low amount of cadmium compared to gills and intestine (Szebedinszky *et al*., 2001; McGeer *et al*., 2012). Water chemistry variables influence cadmium accumulation and toxicity. Dissolved organic carbon and calcium appreciably suppressed gill accumulation of cadmium (Niyogi *et al.,* 2008). Presence of increased calcium ions in the water diminishes cadmium toxicity (Ghosh and Adhikari 2006). In zebra fish, *D. rerio*, zinc decreased uptake of cadmium through branchial epithelium and inhibited influx of cadmium to the circulatory system (Glynn, 2001).

Gupta and Rajbanshi, (1988) reported collapsing of pillar cells, rupture and detachment of lamellar epithelium and hypertrophied mucus gland cells in the gills of *C. punctatus* under acute cadmium poisoning. Accumulation of debris and necrosis in

the renal tubules as well as vacuolisation and necrosis in hepatic cells were observed in the same study. Authman *et al*. (2015) observed that high concentration of cadmium induced suffocation in fishes. Uptake of ions through gills enables fishes to maintain ion homeostasis in the body (Galvez *et al*., 2006; Hwang *et al*., 2011). In teleost, *Oreochromis mossambicus* water borne cadmium affected calcium and magnesium metabolism (Pratap *et al.,* 1989). In European flounder, *P. flesus* the plasma concentrations of magnesium and inorganic phosphate were considerably increased while plasma concentration of potassium and calcium diminished under the influence of cadmium (Larsson *et al.,* 1981). Primary cause of acute cadmium toxicity lies in disrupted ionic balance especially on the impact on calcium homeostasis (Niyogi and Wood, 2004; McGeer *et al*., 2012).

Tolerance to the toxic effect of ubiquitous environmental pollutants mercury and cadmium are diverse among fishes. Olsson *et al*. (1998) suggested that heavy metal tolerance partly depend on the rate of its accumulation and largely on the detoxification mechanisms. Antioxidants like glutathione involves in the detoxification of heavy metals (Gudbrandsen *et al*., 2007; Engström, 2011). Heavy metal toxicity can also induce stress proteins like heat shock proteins in fish tissues (Iwama *et al.,* 1998, Roberts *et al.,* 2010; Mahmood *et al*., 2014). Mechanisms like synthesis of metallothionein, decreased uptake, increased excretion and redistribution of metals to less susceptible targets might decrease cadmium toxicity (Klaassen *et al*., 2009; Kovarova *et al*., 2009; Wang *et al*., 2014; Sandbichler and Höckner, 2016).

Although similar pattern of cadmium accumulation exhibited in testes and ovaries of *R. dandia*, testes did not show further increase in accumulation of mercury after 20 days. However, ovaries showed to continue to accumulate mercury even after

20 days of exposure period (figures 3.3 and 3.4). Also, the high accumulation of mercury in the twenty days of exposure (figure 3.3), shows that the reproductive organs are vulnerable to the initial period of exposure to the heavy metals. Madenjian *et al.,* (2016) found that females accumulated more mercury than males in summer flounder, *P. dentatus* and proposes males may be eliminating mercury faster than females. Kumar *et al.,* (2015) found that zinc bioaccumulation in ovary was comparatively more than in the testis. Mohanambal and Puvaneswari, (2013) observed a gender specific accumulation, where ovaries accumulated more lead than the testis. The Bioaccumulation factor (Bf) of the fish shows that Bf is higher in the case of mercury than in the case of cadmium (table 3.4). Gender specific heavy metal accumulation in gonads may be attributed to various physiological differences in gonad development and require further scientific investigation.

Ebrahimi and Taherianfard, (2010) assessed concentration of heavy metals in the organs of two species of cyprinid fish in the Kor River in Iran. Significant reduction in the amount of progesterone and testosterone in males and estradiol in females was observed where high levels of metals were found in the testes and ovaries correspondingly. Tilton *et al.,* (2003) observed significant reduction in gonadal steroid release in Japanese medaka exposed to cadmium. Toxicants influencing the reproduction can contribute to reduced population size and even result in species extinction.

Heavy metals accumulated by previous generation can induce toxicant stress in fishes. Stefansson *et al.,* (2014) traced maternal methyl mercury using an enriched stable isotope and found that mercury content of eggs can be predicted from clutch size and maternal body burden. Mercury stored in the ovaries may be mobilized into

embryos through eggs resulting in the sharing of maternal burden of mercury by developing embryos. Apart from the maternal transfer, accumulation of mercury can occur in eggs and embryos though bioconcentration of mercury from the surrounding water (Crump and Trudeau, 2009). Cazan and Klerks (2014) studied maternal transfer of cadmium in live-bearing fishes *Gambusia affinis* and *Heterandria formosa* and developing embryos acquired maternal cadmium in both species.

Accumulated toxicants may disrupt the normal physiology of the organism. Xenobiotic toxicants interrupt functioning of the reproductive endocrine system by targeting not only the gonads but also liver and hypothalamus- pituitary axis. Interference at any of these organs alters the rate of gonadal development and gamete quality (Kime and Nash, 1999). Liver is a site of mercury accumulation and involves in methyl mercury elimination (Kidd and Batchelar, 2012). Although various internal organs readily absorb plasma cadmium, liver has the highest absorption among them (McGeer *et al*., 2012). Liver plays a key role in regulating reproduction of fishes. Fish liver synthesises vitellogenin which is later transported to ovaries and incorporated in the eggs. Catabolism of plasma steroids secreted by gonads is aided by liver (Kime, 1999). Accumulated heavy metals in liver impair its functions and may disrupt reproductive physiology as liver is critical organ in fish reproduction. Thus, bioaccumulation of heavy metals can directly and indirectly influence reproduction of wild fish populations, adversely affecting healthy population size and contributing to extinctions of fish species.

CHAPTER 4

EFFECTS OF HEAVY METALS ON THE SEXUALLY MATURE GONADS

Introduction

Fishes are the most important vertebrate species of the aquatic world. Most of the fishes are positioned in the top of the aquatic food web and hence, the fishes are the biotic form which is mostly affected by the pollutants. Several reports have documented that the xenobiotics introduced into the environment by anthropogenic activity can affect the reproductive process in fishes (Arukwe and Goksøyr, 1998). Xenobiotic effects of the pollutants often adversely affect fish reproduction and directly cause massive damage to the fish populations (Islam and Tanaka 2004).

Normal physiology and homeostasis of organisms are often disrupted by non essential metals like cadmium and mercury in the polluted ecosystems. Even at low concentration, mercury may affect fish populations through impairment of physiological processes like reproduction (Crump and Trudeau, 2009). According to Wiener (2013), extend of issues of mercury in fish and wildlife are underestimated and has a scope of substantive scientific discovery. Kidd and Batchelar (2012) pointed out that it is not well understood how long mercury exposure affects the reproductive success of wild fish. Although reproduction is a sensitive endpoint in fish, a few comprehensive studies exist on the impact of cadmium exposure on fish reproduction (McGeer *et al*., 2012).

The aquatic ecosystems are severely affected from global pollution because the final destination of pollutants released elsewhere on the earth is hydrosphere. Often chemicals are disposed into water bodies without sufficient toxicological testing all over the world. Xenobiotic chemicals interfere with the normal physiological processes of aquatic organisms leading to early mortality and sterility. Wild fish populations are ever declining and many fish species are on the verge of extinction.

Developing strategies for identifying chemicals causing damage to organisms is a necessity in the modern era. Understanding on modes of action of toxicants make the prediction of their effects as pollutants more accurate (Ashauer and Jage, 2018).

One of the most sensitive chronic or sublethal effects of pollutants is on reproduction (Javed and Usmani, 2015). According to Kime, (1999) fishes are ideal model organisms to study xenobiotic effects on vertebrate reproduction. Fishes are particularly susceptible to environmental contamination as they have a complex reproductive physiology involving the coordinated action of different tissues (Segner, 2011). Conclusive studies on reproductive dysfunction of major xenobiotic chemicals are inevitable. Apart from identifying harmful chemicals, strategic environment monitoring programmes can also be developed from these studies.

In addition to the reproductive endocrine disruption, heavy metal exposure also induces some pathological changes (Ebrahimi and Taherianfard, 2011). Although histology can be a powerful tool in assessing reproductive health of fishes, a great deal of field and laboratory research, is required to interpret the histological observations obtained by field studies to assess reproductive success of fish populations (Blazer, 2002).

Kumar *et al*., (2007) studied the effect of linear alkyl benzene sulphonate on the testis of fresh water fish, *H. fossilis*. Along with inflammatory response and cytotoxic damage, other histopathological alterations were observed in the treated fishes. Intertubular vacuolation, damage of germinal epithelium and condensation of spermatogenic cells were observed. Shukla and Pandey, (1984a) observed significant changes in the testis of *Colisa fasciatus* by exposure to 14 mg/L of arsenic in water.

They further observed that, lobules were degenerated and diameter of Leydig cells reduced in exposed fishes.

Vergilio *et al*., (2013) studied the effect of mercuric chloride on the testes of tropical fish *Gymnotus carapo*. Significant change in GSI was not observed between control and treated fishes. Pathological changes like proliferation of interstitial tissue, disorganization of seminiferous lobules, congestion of blood vessels and sperm aggregation were observed in mercuric chloride treated fishes. Germ cells and spermatozoa were also reduced in test fishes. Wester and Canton, (1992) observed degeneration and necrosis of sperm in the testis of methyl mercury chloride exposed, *Poecilia reticulata*. Absence of mature sperms, hypertrophy of sertoli cells and interstitial inflammation were noted in severe cases. In *C. carpio*, reproductive functions are influenced by safe concentrations of mercuric chloride (Sahar *et al*., 2009) which shows the deleterious effects of heavy metals on reproductive organs even at low levels of concertation.

Yön *et al*., (2015) investigated the effect of cadmium in the testis of sword tail fish, *Xiphophorus helleri*. Cadmium was found to cause degeneration of seminiferous tubules as well as clumping in spermatocytes. Kumari and Dutt, (1991) exposed *P. sarana* to cadmium chloride and observed disorganization of testicular lobules.

Nath and Kumar, (1990) studied histopathology of gonads of *C. fasciatus* after nickel exposure. Congestion of blood vessels, degeneration of germ cells and rupture of lobules were observed in the testis of nickel intoxicated fishes. Several oocytes underwent atresia and large interfollicular space formed by the shrinkage of oocytes seen in the ovaries after nickel exposure.

Sublethal hexavalent chromium exposure arrested follicular development and increased the percentage of atretic follicles in *C. punctatus*. GSI and ovary wall diameter were also declined under chromium exposure (Mishra and Mohanty, 2012).

Bano and Hasan, (1990) observed reduction in ooplasm, increased oocyte atresia and obvious interfollicular spaces in the ovaries of cat fish, *H. fossilis* by mercury intoxication. Masud *et al*., (2009) observed inhibition of vitellogenesis as well as degeneration of oogonials and immature oocytes in the ovary of *C. carpio* after intoxication with mercuric chloride. Nuclear and ooplasmic dissolution were the degenerative alterations in the oogonials and immature oocytes and debris accumulated in the ovary.

Sharma *et al*., (2011) investigated the histopathological changes in the ovaries of *Heteropnuestes fossilis* induced by cadmium chloride. At 6 mg/L cadmium chloride concentration, enlarged oocytes appeared after 15 days and egg envelope degenerated by 30 days. Nucleoli appear scattered and atretic follicles were found by 45 days. Ovary wall got thinned and ruptured under 9 mg/L for 15 days. Interfollicular spaces were enlarged by 30 days and degeneration of egg envelopes occurred by 45 days, under same concentration of cadmium chloride.

Gonadosomatic index (GSI) is used as an essential parameter to study the reproductive biology of fishes. GSI is a simple measure of the extent of the reproductive investment for gonadal development (Cubillos and Claramount, 2009). GSI also assess the level of ripeness of ovary, and useful to understand the seasonality of reproduction in fishes. A few previous studies showed that environmental contaminants may have direct influence on GSI and gonadal morphology of the fish (Lambert *et al*., 2003; Sakamoto *et al*., 2003). In the present study, the gravid fish, *R*.

dandia was exposed to various levels of heavy metals, mercury and cadmium to understand its influence on GSI.

Most of the literature regarding the effects of pollutants on the reproduction of fishes is limited to a few teleost fishes like *Clarias batrachus, C. punctatus, C. fasciatus, H. fossilis, and Sarotherodon mossambicus* (Pandey, 2000). The present study is an attempt to evaluate the histopathological changes in the gonads of *R. dandia* induced by mercury and cadmium.

Materials and methods

Test organism

Gravid male fishes (*R. dandia*) with 5.5 to 7 cm total length and 1.5 to 4.5 gm weight and gravid female fishes with 7 to 8.5 cm total length and 2.5 to 5.5 gm weight were selected. The physico chemical parameters of the water in the aquaria is maintained as described in chapter 2. The aquaria were well aerated as described previously and fishes were fed with commercially available standard fish feed. Gravid males which ooze out milt and gravid females which release eggs on slight pressure on the abdomen were selected for experiments.

Test groups, exposure procedure and sampling

Mercuric chloride (Loba chemie, India, extra pure grade) and cadmium chloride (Merck India, analytical grade) were used as the source of mercury and cadmium respectively for exposing gravid fishes to heavy metal stress. Three experimental group consisting of ten fishes of each sex of *R. dandia* were maintained and one group was maintained as control group. One experimental group of fishes of each sex were exposed to sublethal concentration of 0.01 ppm of mercury and the other for 1 ppm of cadmium for 10 days, 20 days and 30 days. Test solutions were

renewed every 24 hours and the mercury and cadmium solutions were added to the aquaria to maintain the respective concentrations. Fishes of each sex were selected at random, anesthetized and sacrificed on every ten days' time interval during the experiment. Each experiment was repeated for three times, following the above protocol.

Histological preparation

Exposed gravid male and female fishes of each test groups were selected at random and the gonads were dissected out carefully. The adhered water was blotted off using Whatman filter paper. The gonads were fixed and histological slides of testes and ovaries were prepared as described in chapter 2 of the thesis and histopathological changes were noted. The images of the sections were taken as described in chapter 2 of the thesis.

Estimation of Gonadosomatic index (GSI)

To estimate the GSI, five male and female fishes were selected at random from each test group. The fishes were anesthetized, sacrificed and gonads were dissected out carefully. The water adhered to the testes and ovaries are blotted off and weighed using a sensitive electronic balance (Shimadzu, Japan). In a similar manner, the wet body weight of the somatic mass of the fish was taken after washing and blotting off the adhered water. GSI is expressed as gonadal mass percentage of total body or somatic mass and calculated according to the formula (gonadal weight/body weight) X 100 (Valdés *et al*., 2004; Zadmajid *et al*., 2017). The experiment was repeated for each sex for three times.

Results

GSI was found to be slightly declined by 20 days of mercury exposure, among the male fishes. By 30 days of mercury exposure GSI of male fishes reduced considerably. GSI was found to be progressively declined as the exposure period of mercury increases in female fishes (table 4.1). Cadmium exposure caused progressive decline of GSI of male fishes as the exposure period increases. In cadmium exposed female fishes, significant reduction in GSI occurred only by 30^{th} day of exposure (table 4.2).

Table 4.1: Effect of mercury exposure on GSI of *R. dandia* (Mean± SD). Results are the mean of three replicates of each group with standard deviation. (Significant at $p<0.05$* Significant at $p<0.01$** Significant at $p<0.001$***; Student's t-test).

Exposure period	Male control fishes	Male fishes exposed to 10 ppb mercury	Female control fishes	Female fishes exposed to10 ppb mercury
10 days	6.3 ± 0.64	6.06 ± 0.389	19.02 ± 0.98	12.47 ± 1.17**
20 days	6.29 ± 0.14	5.47 ± 0.38*	19.73 ± 1.35	7.29 ± 0.38**
30 days	6.47 ± 0.12	2.56 ± 0.43**	19.28 ± 0.67	4.19 ± 0.23***

Table 4.2: Effect of cadmium exposure on GSI of *R. dandia* (Mean± SD). Results are the mean of three replicates of each group with standard deviation. (Significant at p<0.01* Significant at p<0.001**; Student's t-test).

Exposure period	Male Control fishes	Male Fishes exposed to 1 ppm cadmium	Female Control fishes	Female Fishes exposed to 1 ppm cadmium
10 days	6.08 ± 0.29	4.42±0.48	19.38 ± 0.76	19.05 ± 0.51
20 days	6.57 ± 0.59	2.44 ± 0.25*	19.58 ± 0.99	18.20 ± 0.56
30 days	6.04 ± 0.64	1.66 ± 0.28*	19.06 ± 0.82	12.09 ± 0.95**

In control samples, testis of *R. dandia* showed germ cells at different stages of maturation. Most abundant among them was spermatozoa. Lobules were filled with large number of spermatozoa with small clusters of spermatids and spermatocytes adjacent to the interstitial tissue (figure 4.1). However histological alterations were observed in the testis of *R. dandia* when exposed to mercury. Vacant spaces increased inside the lobules by 10 days of mercury exposure (figure 4.2). After 20 days mercury exposure interstitial tissue appeared to be proliferated and ripe spermatozoa started declining (figure 4.3). Bundles appeared disorganised, vacant spaces enlarged and considerable reduction in spermatozoa occurred by 30 days of mercury exposure (figure 4.4).

By 10 days of cadmium exposure abundance of ripe spermatozoa appeared to be declined (figure 4.5). Vacant spaces increased inside lobules due to this disappearance of spermatozoa by cadmium exposure (figure 4.7). In the testis of 20 days cadmium treated fishes, some areas showed disorganised lobules (figure 4.6). Detachment of basement membrane occurred in the testis of cadmium exposed fishes at 30 days (figure 4.8). Blood vessels appeared to be dilated and collapsed by 30 days

of exposure. This resulted in the invasion of nucleated ellipsoid red blood cells into lobules (figure 4.7).

Histopathological changes appeared in the ovary of the fish from 10 days of mercury exposure (figure: 4.10). Mass atresia of oocytes occurred resulting in huge loss of oocytes in the ovaries by 20 days of mercury exposure (figure 4.11). On the 30^{th} day, it is observed that most of the vitellogenic oocytes were destroyed and immature oocytes become predominant in the ovary (figure 4.12). Ripe ovaries transformed in to shrunken immature ovaries with histopathological alterations (figures 4.9, 4.10, 4.11, and 4.12). Among immature oocytes, which undergoing atresia, large vacuoles were formed and invaded to the nucleus. Oocyte membranes were convoluted following yolk resorption in atretic mature oocytes (figure 4.11). Fibrotic tissues appeared in the areas where massive oocyte loss occurred in the ovaries (figure 4.12).

No histopathological changes were observed in the ovaries of fishes up to 20 days of cadmium exposure (figures 4.9, 4.13 and 4.14). Areas of mass cellular atresia were observed in the ovary of fishes exposed to cadmium by 30 days of exposure (figure 4.15).

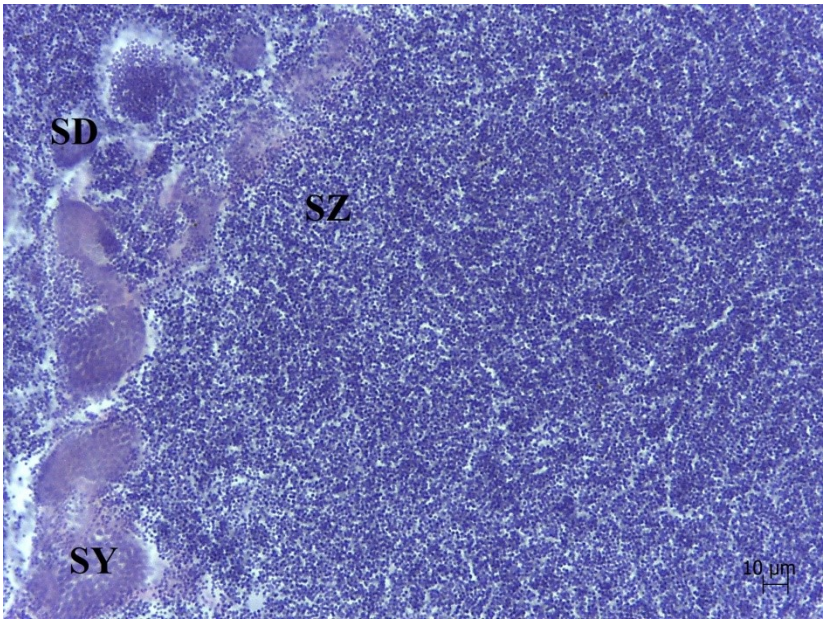


Figure: 4.1, Histological section of testis of *R. dandia* (control), (H&E; x 400); SZ- Spermatazoa, SY- Spermatocytes, SD- Spermatids.

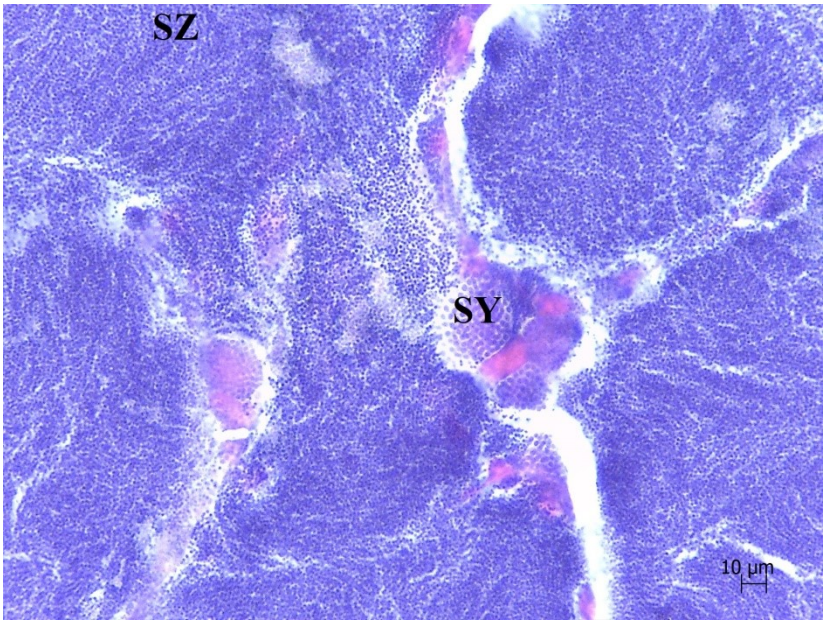


Figure: 4.2, Histological section of testis of *R. dandia* exposed to mercury for 10 days, (H&E; x 400); SZ- Spermatazoa, SY- Spermatocytes.

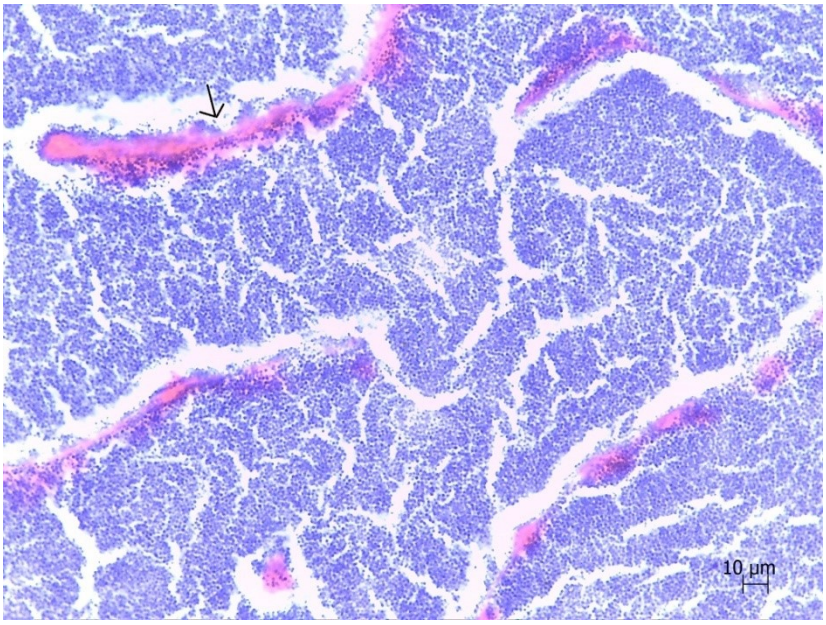


Figure: 4.3, Histological section of testis of *R. dandia* exposed to mercury for 20 days, (H&E; x 400); Proliferation of interstitial tissue (→).

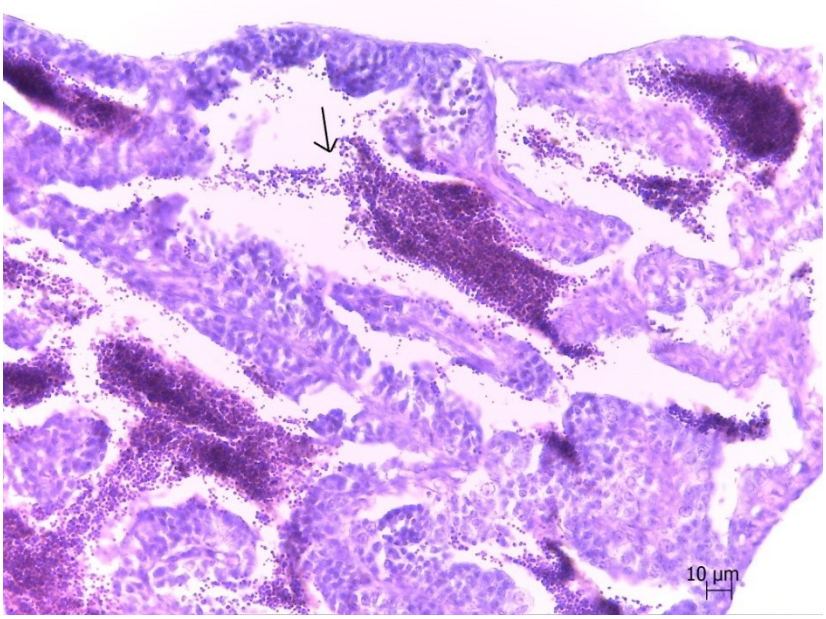


Figure: 4.4, Histological section of testis of *R. dandia* exposed to mercury for 30 days, (H&E; x 400); Reduced spermatozoa (→).

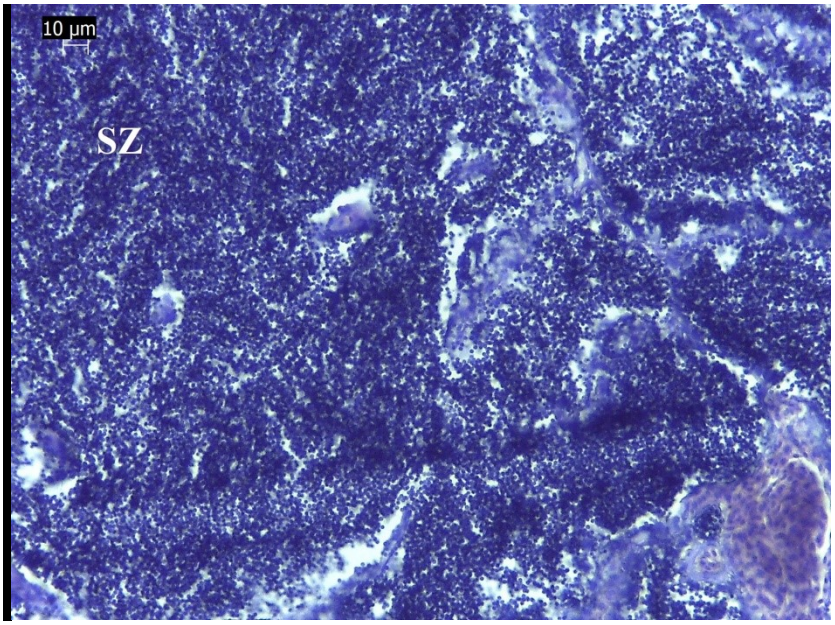


Figure: 4.5, Histological section of testis of *R. dandia* exposed to cadmium for 10 days, (H&E; x 400); SZ- Spermatazoa.

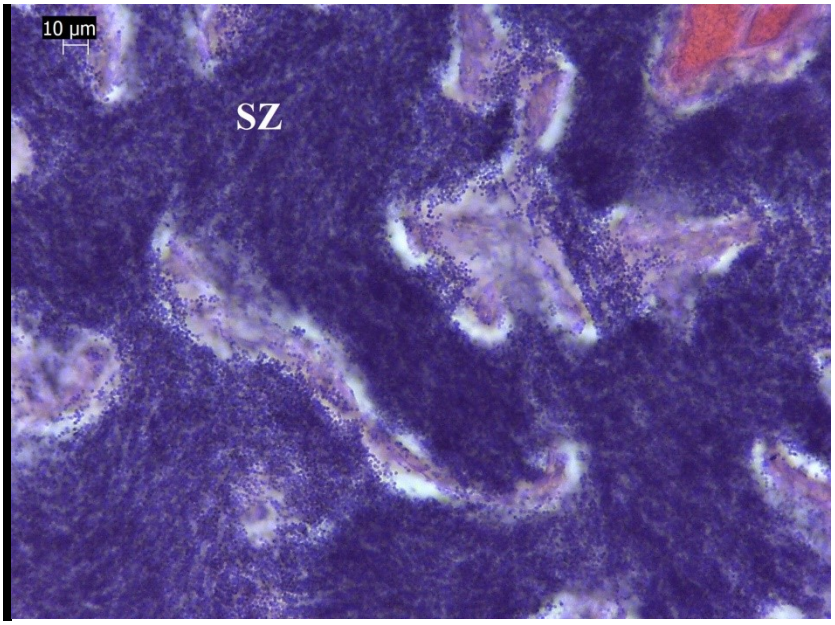


Figure: 4.6, Histological section of testis of *R. dandia* exposed to cadmium for 20 days, (H&E; x 400); SZ- Spermatazoa.

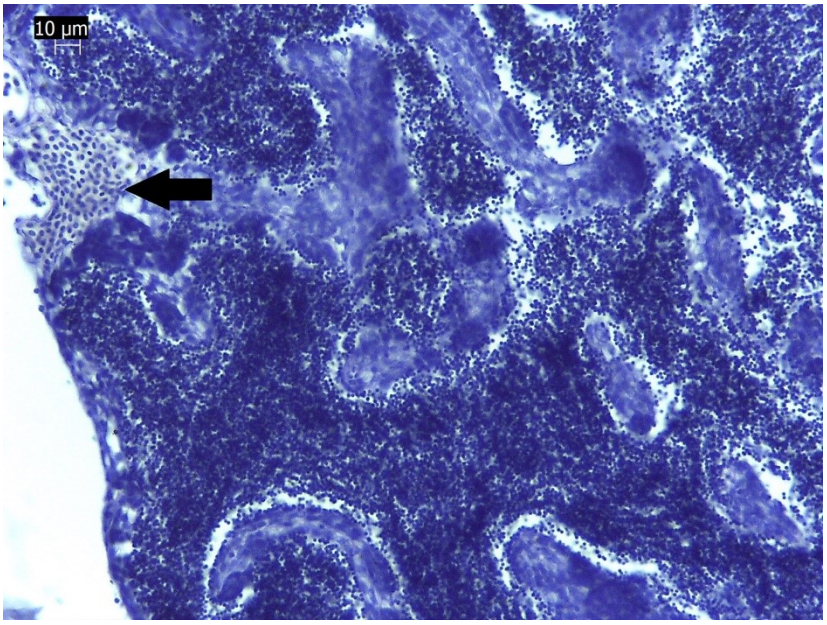


Figure: 4.7, Histological section of testis of *R. dandia* exposed to cadmium for 30 days, (H&E; x 400); Dilated blood vessel (➡).

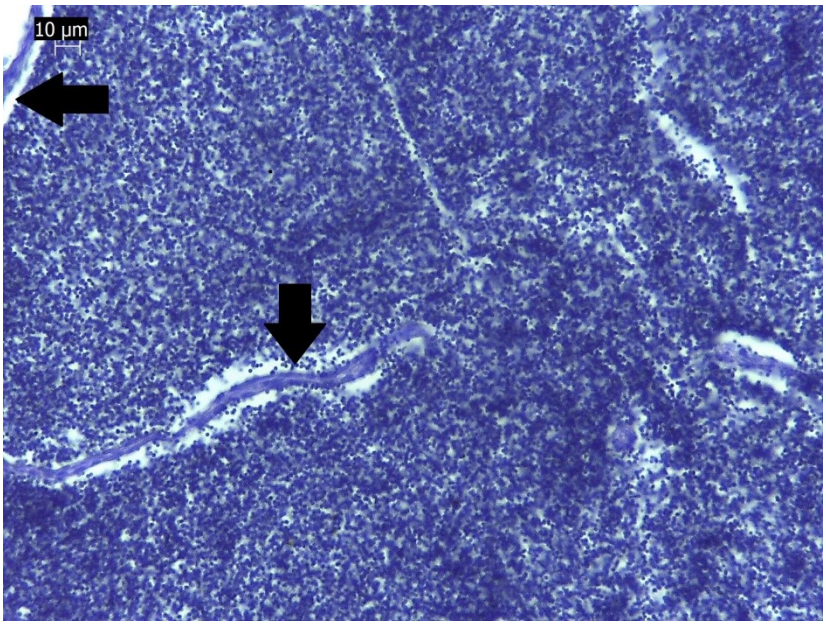


Figure: 4.8, Histological section of testis of *R. dandia* exposed to cadmium for 30 days, (H&E; x 400); Detached basement membrane (➡).

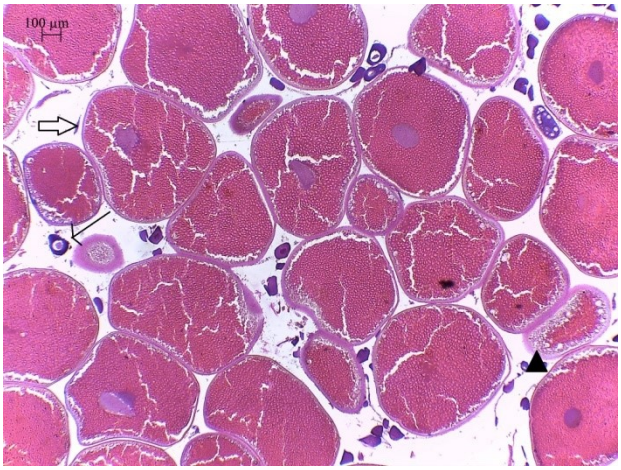


Figure: 4.9, Histological section of ovary of *R. dandia* (control) (H&E; x 40);

Immature oocyte (→), Maturing oocyte (▲), Mature oocyte (⇨).

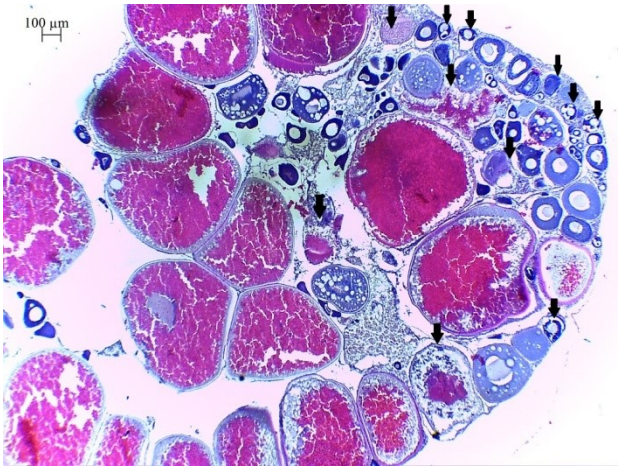


Figure: 4.10, Histological section of 10 days Hg exposed ovary of *R. dandia*, (H&E; x 40); Large number of cells started undergoing atresia (➡).

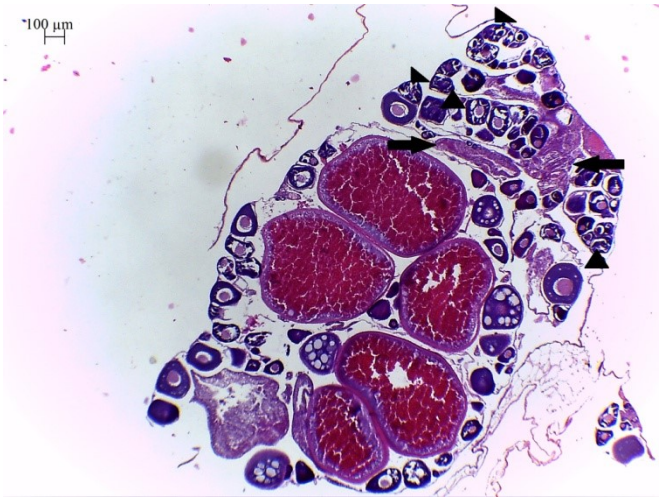


Figure: 4.11, Histological section of 20 days Hg exposed ovary of *R. dandia*, (H&E; x 40); Atresia in immature oocyte (▲), Atresia in mature oocyte (➡).

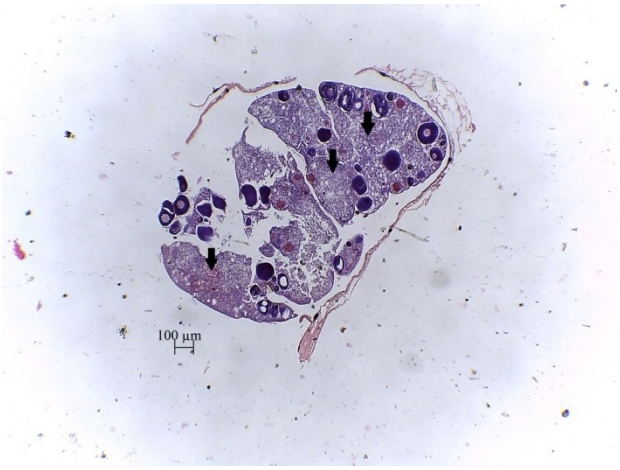


Figure: 4.12, Histological section of 30 days Hg exposed ovary of *R. dandia*, (H&E; x 40); Areas devoid of oocytes (➡) appeared in the ovary.

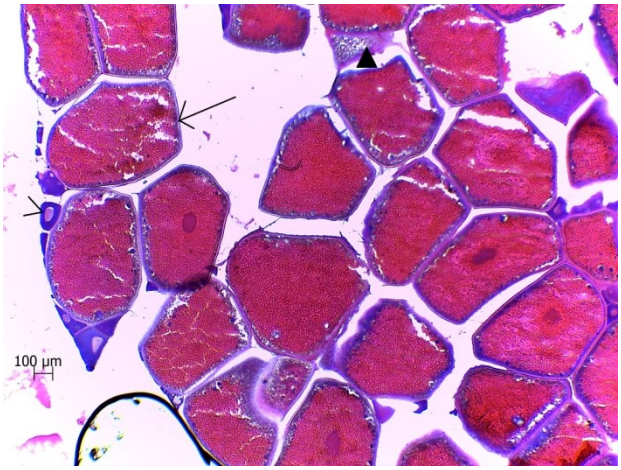


Figure: 4.13, Histological section of 10 days Cd exposed ovary of *R. dandia*, (H&E; x 40); Immature oocyte (>), Maturing oocyte (▲), Mature oocyte (→).

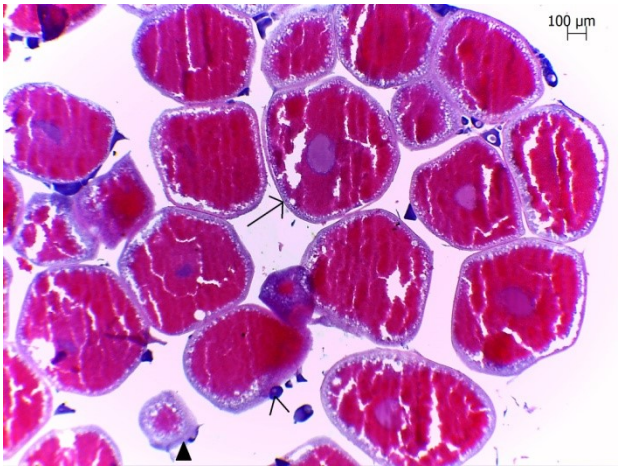


Figure: 4.14, Histological section of 20 days Cd exposed ovary of *R. dandia*, (H&E; x 40); Immature oocyte (>), maturing oocyte (▲), mature oocyte (→).

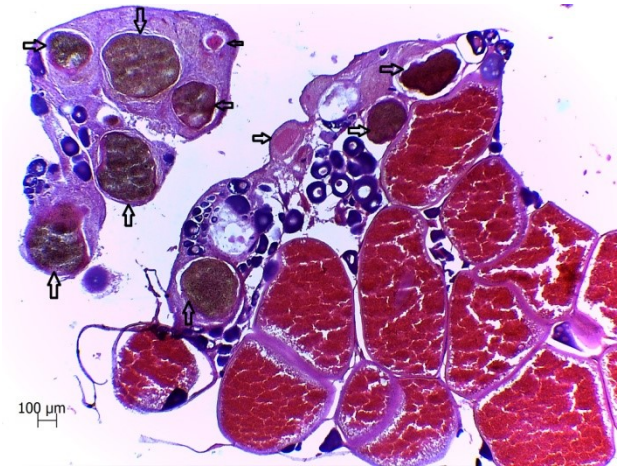


Figure: 4.15, Histological section of 30 days Cd exposed ovary of *R. dandia*, (H&E; x 40); Atretic oocyte (⇨).

Discussion

Testis and ovary are the primary reproductive organs of teleost which are essential for gametogenesis. They are composed of various stages of germ cells, interstitial cells and supporting cells (Nishimura and Tanaka, 2014). In seasonally breeding fishes, prior to the onset spawning, gonads develop in to a gravid sexually mature condition with considerable increase in size and the presence of abundant ripe gametes. The present study observed the detrimental effects of heavy metal exposure on the sexually mature gonads of *R. dandia.*

According to Flores *et al*., (2015), GSI is an indicator of the state of the gonads. GSI gives an assessment of reproductive potential of fishes. In fish biology, it is widely used as a measurement of reproductive allocation and reproductive condition (Trindade-Santos and Freire 2015). According to Kime (1999), GSI is the simplest method to know gonadal dysfunction as reduced GSI indicates decreased activity of reproductive axis.

The present study showed that GSI of the test fishes is affected by mercury exposure, but severe reduction occured only by 30 days of exposure period (table 4.1). Kirubagaran and Joy (1992) observed a significant decrease in the GSI of male *C. batrachus* exposure to the mercurials. In the present study, GSI of male fishes are found to be gradually decreased by cadmium exposure (table 4.2). Vergilio *et al*., (2015) observed reduced GSI along with increased morphological alterations in the tropical fish *G. carapo* exposed to cadmium concentrations higher than 10 µM. In the present study, GSI of female *R. dandia* gradually declined under the influence of mercury where as in cadmium exposed female fishes GSI declined by 30 days of exposure (tables 4.1 and 4.2).

Mercury exposed test fishes showed histopathological changes in the testis which gradually worsened with increase of exposure period (figures 4.2, 4.3 and 4.4). Vergilio *et al.*, (2013) reported that mercury induced effects on the testis of *G. carapo* became more severe with increase of time. Severe deterioration was observed in the testicular histology with proliferation of interstitial tissue and congestion of blood vessels. Proliferation of interstitial tissue was observed in the testis of fishes exposed to mercury in the present study (figure 4.3) supports the results of Vergilio *et al*. (2013).

Severe deterioration in testis structure was observed by 30 days of mercury exposure like disorganised bundles, enlarged vacant spaces and significant reduction in spermatozoa (figure 4.4). Ram and Sathyanesan (1983) observed that the exposure to mercuric chloride stopped spermatogenesis in teleost fish, *C. punctatus*. According to Crump and Trudeau (2009), testicular impairment by mercury exposure is attributed to its direct cytotoxic effects as well as disruption of endocrine function. The results of the present study indicate that the presence of mercury in water can induce substantial testicular damage in fishes.

When male *R. dandia* was exposed to cadmium for 30 days, blood vessels of testis appeared damaged resulting in the invasion of red blood cells into lobules (figure 4.7). Similar observations were carried out by earlier investigators. In *P. Sarana,* Kumari and Dutt (1991) observed disrupted vascularisation and haemorrhagic necrosis in testis when exposed the fish to cadmium chloride. Rajan and Kuzhivelil (2015) reported the collapse of blood vessels and also found that red blood cells were surrounding the lobules in testis

of cadmium exposed *R. dandia*. The results of the present study indicates that exposure of cadmium induce damages in the testis of fishes.

In fishes, oocytes in immature stage, mature through yolk vesicle accumulation (Tyler and Sumpter,1996). Various detrimental alterations can occur in the ovaries by the influence of xenobiotic toxicants in the normal physiological functions of gonads. Advanced phases of oocytes were destroyed and dominance of immature oocytes observed in the ovary of *C. punctatus* by mercury exposure (Dey and Bhattacharya, 1989). Decrease in oocyte development was observed in *C. carpio* after exposure to safe concentration of mercuric chloride for short duration (Masud *et al*., 2009). Impaired vitellogenesis, reduced GSI and degeneration of oocytes were observed in the ovaries of *C. batrachus* exposed to Emisan 6, methyl mercuric chloride and mercuric chloride by Kirubagaran and Joy (1988). The results of the present study shows that the exposure of mercury to fishes may transform ripe ovaries into shrunken immature ovaries with histopathological alterations (figures 4.9, 4.10, 4.11 and 4.12). The study proves the opinion of Field (1998) that reproductive toxicity is a sensitive endpoint with potentially significant implications for the health of fishes.

In the present study, massive loss of oocytes by atresia occurred in the ovary of *R. dandia* by 30 days of mercury exposure (figure 4.12). Degeneration of oocytes may impair number and viability of fish progeny. Sensitivity to mercury to different phases of oocytes is detrimental to the reproductive success of teleost fishes.

Fibrotic tissue appeared in place of lost oocytes by thirty days of mercury exposure in the ovary of *R. dandia* (figure 4.12). Under mercury exposure, infiltration of fibroblast like cells in the ovary of *C. batrachus* was observed by Kirubagaran and Joy (1988). Irreversible ovarian damage by fibrosis and massive loss of oocytes under

the influence of mercury were evident in the present study (figure 4.12). The results indicate that in the case of acute exposure of mercury, irreversible changes in the gonads may occur resulting in sterile populations. Masud *et al.,* (2009) observed a limited recovery response in the ovary after short term exposure to safe concentration of mercuric chloride. They observed recruitment of new immature oocytes, possibly from unaffected oogonials. However, they found that irrevocable damage occurred in ovary by cellular debris material present near normal immature oocytes. The present study proves the observations in the earlier studies (Kirubagaran and Joy, 1988; Dey and Bhattacharya 1989; Masud *et al*., 2009) and shows that even short episodes of mercury influx in aquatic ecosystems impair reproductive potential of organisms.

Sharma *et al*., (2011) observed atretic follicles in the ovaries of the air-breathing fish, *H. fossilis* after 45 days of cadmium exposure. Increased proportion of atretic follicles was reported in the ovary of zebra fish exposed to cadmium by Chouchene *et al.,* (2011). These results support the observation of mass atresia in the ovary of *R. dandia* after thirty days of cadmium exposure (figure 4.15). Although atresia is a normal physiological event, increased follicular atresia of the fish ovaries in response to toxicant stress was indicated by several earlier studies (Kumar and Pant, 1984; Shukla and Pandey, 1984b; Mani and Saxena, 1985; Pandey, 1988; Dutta and Dalal, 2008; Marutirao, 2013). The mass atresia observed in the present study supports the above studies.

Reduction in GSI as well as reduction in the number of mature gametes indicates reduced reproductive success. Some histopathological alterations may persist even after the cessation of toxicant influx, leading to permanent sterility. Compromised reproductive potential under toxicant stress reduce the population size of sensitive species. Apart from causing mass mortalities and reduced lifespan,

environmental toxins may induce extinction of species also by interfering in normal reproductive physiology of organisms. Physiological impacts of mercury on fishes may similarly arise in piscivorous vertebrates as fishes are their food source. Mercury indirectly affects the top predators by reducing food supply while directly affects them by concentrating in the food chain (Crump and Trudeau, 2009).

According to Schreck (2010), reproduction has narrow tolerance to stress than other life functions and indicated the requirement of adequate research on the effects of stress on the reproductive performance of fishes. Increased stress sensitivity of reproductive system makes it ideal location for the search of biomarkers of stress. Atretic oocytes were found to be elevated in the ovary of *Micropterus salmoides* along with the increase of sediment mercury (Adams *et al*., 1999). Taniconi *et al*., (2015) studied gonadal alterations in thin lip grey mullet from polluted river sites and proposed it as a bioindicator for evaluating the health of aquatic ecosystems. Histopathologic biomarkers like elevated oocyte atresia can be connected to the impact of toxicants on population (Hinton *et al*., 1992; Hinton, 1993; Chiang *et al*., 2013). The observations in the present study on gonadal alterations like mass atresia, fibrosis and reduced number of mature oocytes under the stress of heavy metal offer it as suitable candidates for histopathological biomarkers. However, laboratory studies of heavy metal toxicity should be supplemented with sufficient correlational field studies to develop these parameters as biomarkers of mercury stress in aquatic environment.

Apart from the controlling of heavy metal emissions into the environment, the need for constant biomonitoring of pollution prone ecosystems is also rising. Reproductive impairment can be used for devising biomonitoring programmes if sufficient data are provided on the reproductive toxicity of pollutants of major

ecological concern. Zhou *et al*., (2008) suggests that biomonitoring is an appealing tool for the assessment of ubiquitous metal pollution in aquatic ecosystem. According to Hutchinson *et al*., (2006) secondary sexual characteristics, GSI and gonadal histology are significant biomarkers for guiding interspecies assessments. Thus, toxicological data on the effect of major aquatic pollutants on different fish species provide sufficient comparative data facilitating the development of biomarkers.

CHAPTER 5

EFFECTS OF HEAVY METALS ON THE GONADAL RECRUDESCENCE

Introduction

Seasonal breeding is a common reproductive strategy among tropical fishes (Winemiller, 1993; Ballesteros *et al.,* 2009; Chellappa *et al.,* 2009; Rangel-Serpa and Torres, 2015). Reproductive seasonality of tropical fresh water fishes largely depends upon hydrological cycle with a strong cue from rainfall (Wootton, 1998; Rangel-Serpa and Torres, 2015). Cyprinoids, a major group among tropical fresh water fishes which are generally highly seasonal in reproduction focusing on the floods formed by early rainy season (Lucas and Baras 2001). This may be correlated to scheduling reproductive effort to periods ideal for juvenile growth and not synchronized with increased availability of adult food resources (Winemiller, 1993; Ballesteros *et al.,* 2009).

Endogenous physiological mechanisms of gonadal maturation are synchronized with environmental factors in seasonal reproduction of fishes (Mishra and Sarkar, 2013). In cyprinids temperature and photoperiod are reported to play a significant role in regulation of reproductive events although the pattern varies among different species (Okuzawa *et al.,* 1994; Acharia *et al.,* 2000).

Gonadal recrudescence is the revival of regressed gonad into sexually ripe condition ready to undergo breeding. After gonadal recrudescence, GSI will be elevated consisting about 5-10 percentage of body weight in males and 20-30 percentage of body weight in females (Kime, 1998). Gonadal recrudescence is controlled by environmental factors as well as internal physiological mechanisms like endocrine regulation. Reproduction being a sensitive biological function, various environmental contaminants may disrupt gonadal recrudescence of fishes. Xenobiotics can act directly by toxic effects on gametes as well as indirectly through

endocrine disruption or influencing organs like liver or pituitary which involve in reproductive processes (Kime, 1999; Goksøyr, 2006; Scholz and Klüver, 2009).

Sandström *et al.,* (1988) studied reproductive capacity of roach, *Rutilus rutilus* and perch, *Perca fluviatilia* in a coastal area affected by bleached pulp mill effluents of the Bothnian Sea. The number of fishes exhibiting inhibition of gonadal recrudescence was elevated near the mill among perches. The sizes of developing gonads were relatively small. Although found diffused in effect, pattern of reduced gonad growth was found among roaches. Mani and Saxena, (1985) investigated the effect of pesticides, carbofuran and fenitrothion, on the ovarian recrudescence of the teleost *C. punctatus*. Ovarian weight was reduced in pesticide treated fishes than the control fishes. Pesticide treatment affected the growth of previtellogenic oocytes into vitellogenic and yolky stages of oocyte maturation. Percentage of atresia was higher in pesticide treated fishes and only atretic oocytes were found in the ovaries of fenitrothion treated fishes by 120 days. Impact on ovarian recrudescence was more intense in fenitrothion exposed fishes than the carbofuran exposed fishes. Mani and Saxena, (1987) investigated the effect of pesticides, carbofuran and fenitrothion, on the testicular recrudescence of teleost *C. punctatus*. Formation of sperm and spermatids delayed in carbofuran exposed fishes. Apart from delaying the formation of spermatids, fenitrothion arrested sperm formation and cause necrosis of spermatids. Empty lobules devoid of spermatogenetic cells were observed in fishes treated with pesticides.

Kirubagaran and Joy, (1988) studied the impact of methyl mercuric chloride, mercuric chloride and mercurial fungicide, Emisan 6 on the ovarian recrudescence of catfish, *C. batrachus*. Control fishes exhibited normal rate of ovarian growth with

immense increase in the GSI and ovaries were filled with oocytes with yolk globules. Ovarain recrudescence was arrested in test fishes with low GSI and only non vitellogenic oocytes present in the ovary. Ram and Sathyanesan, (1983) studied the influence of mercuric chloride in the reproductive cycle of teleost, *C. punctatus* for six months. All fishes were in the resting stage of gonads during the commencement of the experiment. By the end of experiment, control fishes were in mature phase of gonad development. Control ovaries have majority of the oocytes in preparatory and mature phase, whereas treated fishes had only stage I oocytes. The testes of exposed fishes were in recrudescence stage with secondary spermatogonial cells filled in the tubules. Control testes were in active spermatogenesis with sperms in lumen. Ram and Sathyanesan, (1986) exposed *C. punctatus* to mercurial fungicide, Emisan and observed the arrested gonad development and reduction in GSI of exposed fishes compared to control group. Majority of oocytes completed vitellogenesis and the follicular epithelium was well differentiated in the ovary of control fishes. In the Emisan treated fishes, the ovaries had large number of oocytes in non vitellogenic stage and follicular epithelium was undifferentiated. Remnants of degenerated preovulatory oocytes were present in the ovaries of the exposed group. Control fishes exhibited active spermatogenesis and bundles of sperm in the lumen of testis. The interstitial cells of testis were in active stage with large rounded nuclei and prominent nucleoli in the control group. The interstitial cells were inactive in Emisan treated fishes. Necrotic sites were also appeared in the testis of treated fishes.

Drevnick and Sandheinrich, (2003) studied the effects of dietary methyl mercury on the reproductive success of fathead minnows, *P. promelas*. Juvenile were fed with diets contaminated with methyl mercury and fishes were paired to allow reproduction at sexual maturity. In this study, it was found that plasma testosterone in

males and 17β-estradiol in females were suppressed by methyl mercury. Gonadal development was inhibited and GSI was lower in females fed with methyl mercury contaminated diet. Methyl mercury lowered reproductive success with reduced spawning success.

Studies on the influence of cadmium in the gonad development in aquatic organisms including fishes were limited. Kogan *et al.,* (2000) studied gonadal growth of juvenile female estuarine crab, *Chasmagnathus granulata,* under the influence of cadmium. Revathi *et al.,* (2011) investigated the effect of cadmium chloride on the ovarian development of the eye stalk ablated and intact fresh water prawn, *Macrobrachium rosenbergii.* Kasinathan, *et al.,* (1987) investigated the impact of cadmium chloride on the spermatogenesis of *Rana hexadactyla* by administering single dose of 0.5 mg cadmium chloride as subcutaneous injection.

Szczerbik *et al.,* (2006) exposed goldfish, *Carassius auratus* to 0.10, 1.00 and 10.00 mg cadmium g^{-1} of wet weight of feed. In doses from 0.10 to 1.00 mg Cd g^{-1} of feed, histological analysis and GSI indicated no differences in ovarian development. GSI decreased and ovulation did not occur in fishes treated with 10.00 mg cadmium g^{-1} of feed. El-Ebiary *et al.,* (2013) investigated the influence of dietary cadmium on the reproduction and gonad maturation of red tilapia and the doses applied were 0.25, 0.50 and 1.00 mg cadmium g^{-1} of feed. The highest dose impaired ovarian development and suppressed spawning in female fishes. In males a significant lowering of sperm motility and sperm number occurred under the influence of the same does. 0.25 and 0.50 mg cadmium g^{-1} of feed did not impair gonad development of the exposed fishes. But these doses reduced number of spawned eggs, hatchability and elevated abnormalities in fries.

Thomas, (1989) exposed Atlantic croaker, *Micropogonias undulates* to 1.00 ppm cadmium for seawater up to 40 days and ovarian recrudescence was observed. Cadmium accelerated ovarian growth and raised plasma estradiol level, indicating stimulation of vitellogenesis. Secretion of gonadotropin releasing hormone increased in the pituitaries of cadmium exposed fish. Tilton *et al.,* (2003) exposed adult Japanese medaka, *Oryzias latipes* to 0.00-10.00 ppb of cadmium for seven weeks. Plasma vitellogenin and hepatic estrogen receptor were found unaffected. Gonadal steroid release was lowered in males and females at all cadmium concentrations. At concentrations higher than 5.00 ppb of cadmium, female plasma estradiol levels were changed significantly.

In this study, the effect of heavy metals, mercury and cadmium is evaluated on the gonadal recrudescence of *R*. *dandia*.

Materials and methods

Test organisms and exposure procedure

R. dandia fishes were collected and maintained in the laboratory as described in chapter 2. Grown up male fishes with 5.50 cm to 6.50 cm total length and 1.50 gm to 3.00 gm weight were selected. The selected female fishes for experiments were with total length of 6.00 cm to 7.50 cm and 2.50 gm to 5.00 gm. The fishes were divided into seven groups, each group consisting of thirty fishes with male to female ratio as 1: 1. Three sublethal doses of mercury (1.00 ppb, 2.00 ppb, and 3.00 ppb) and cadmium (0.10 ppm, 0.20 ppm and 3.00 ppm) were selected and one experimental group was maintained as control group without exposure to mercury or cadmium. Test solutions and water in the control group was renewed in every 24 hours and respective level of mercury and cadmium was added. The fishes were fed with commercially available standard fish feed. The exposure period was spanned form

February to May, during these months the active gonad development would take place, before the onset of the breeding season associated with the southwest monsoon. Separate set of experimental groups were maintained for histological study and GSI estimation.

Histological preparation of gonads

Five male and female fishes from each test solutions were selected at random so as to obtain biological indices from random samples. Gonads were dissected out, histological slides were prepared, observed under light microscope and images were captured as described in chapter 2 of the thesis.

Estimation of Gonadosomatic index (GSI)

To estimate the GSI, ten male and ten female fishes were selected at random and the GSI was estimated as described in chapter 4 of the thesis. Significance of GSI was analysed using ANOVA along with Turkey's Post-hoc test with SPSS software 16.0.

Results

At the end of the exposure period in May, control fishes became sexually mature with abundant ripe spermatozoa in the tubular lumen of the testis. Earlier cell stages of spermatogenesis were not evident in control fishes, other than small patches of spermatids seen infrequently (figure 5.1). 0.10 ppm cadmium treated fishes exhibited large and numerous patches of spermatids compared to control fishes. Bundles of ripe spermatozoa appeared less dense in 0.10 ppm cadmium treated fishes (figure 5.2). Among 0.20 ppm cadmium treated fishes' initial stages of spermatogenesis like spermatocytes were very prominent with further reduction in ripe spermatozoa (figure 5.3). 0.30 ppm cadmium treated fishes appeared in very early stages of gonadal recrudescence with limited presence of sperm bundles (figure 5.4). Compared to control fishes, spermatids were more common in the 1.00 ppb mercury treated fishes (figure 5.5). 2.00 ppb and 3.00 ppb mercury treated fishes exhibited large patches of spermatids, along with vacant areas devoid of cells in histological structure of testis (figures 5.6 and 5.7). Ripe spermatozoa appeared less dense in testicular bundles of mercury exposed fishes (figures 5.5, 5.6 and 5.7).

Control fishes exhibited sexually mature ovary with abundant mature oocytes (figure 5.8). 0.10 ppm cadmium and 1.00 ppb mercury treated fishes exhibited areas of mass atresia among immature oocytes (figures 5.9 and 5.12). Mass atresia of immature oocytes was much more severe in 0.20 ppm and 0.30 ppm cadmium treated fishes (figures 5.10 and 5.11). On 2.00 ppb and 3.00 ppb mercury exposure, atresia in mature oocytes also appeared apart from the mass atresia among immature oocytes (figures 5.13 and 5.14). Control fishes exhibited sexually mature ovary with no signs of mass atresia (figure 5.8). Mature oocytes were limited in 3.00 ppb mercury (figure

5.14). Reduction in mature oocytes was evident in 0.20 ppm and 0.30 ppm cadmium treated fishes also (figures 5.10 and 5.11).

Heavy metal treated fishes exhibited lowered GSI compared to control fishes. Reduction in GSI was evident in all concentrations of cadmium and mercury exposure in both sexes (table 5.1 and 5.2). In male fishes, cadmium exposure during gonadal recrudescence caused dose dependent reduction in GSI. Among mercury treated male fishes there was no significant difference in GSI between 1.00 ppb, 2.00 ppb and 3.00 ppb mercury concentrations (table 5.1). 0.10 ppm and 0.20 ppm cadmium treated female fishes did not have significant difference in GSI between them. 0.30 ppm cadmium treated female fishes exhibited further reduction in GSI. Although three concentrations of mercury exposure during recrudescence had reduced GSI in female fishes compared to control, there were no significant differences in GSI between them (table 5.2).

Table 5.1: Effect of heavy metal exposure on GSI of male *R. dandia* (Mean ± SD). Values with different superscript letters vary significantly between exposure groups ((p<0.05; One way-ANOVA, Tukey's post-hoc test).

Group of fishes	GSI
Control	5.83 ± 0.24^{a}
0.1 ppm of cadmium	2.58 ± 0.15^{b}
0.2 ppm of cadmium	2.06 ± 0.10^{c}
0.3 ppm of cadmium	1.54 ± 0.10^{d}
1 ppb of mercury	4.70 ± 0.36^{e}
2 ppb of mercury	4.26 ± 0.69^{e}
3 ppb of mercury	3.82 ± 0.24^{e}

Table 5.2: Effect of heavy metal exposure on GSI of female *R. dandia* (Mean ± SD). Values with different superscript letters vary significantly between exposure groups ((p<0.05; One way-ANOVA, Tukey's post-hoc test).

Group of fishes	GSI
Control	14.22 ± 0.66^{a}
0.1 ppm of cadmium	9.24 ± 0.65^{b}
0.2 ppm of cadmium	9.09 ± 0.61^{b}
0.3 ppm of cadmium	7.46 ± 0.49^{c}
1 ppb of mercury	9.28 ± 0.83^{d}
2 ppb of mercury	9.24 ± 1.04^{d}
3 ppb of mercury	9.15 ± 0.94^{d}

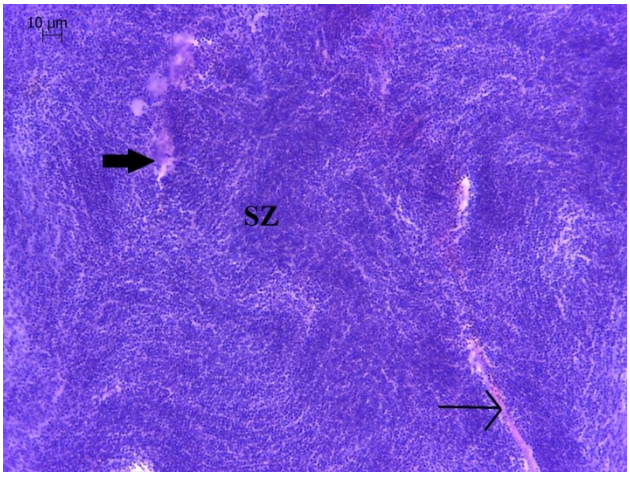


Figure: 5.1, Histological section of testis of *R. dandia* (Control), (H&E; x 400); Spermatozoa (SZ), Spermatids (➡), interstitial tissue (→); Tubules are filled with abundant spermatozoa.

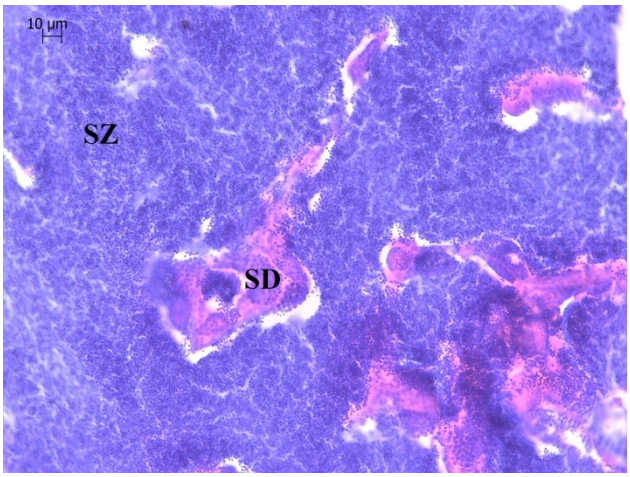


Figure: 5.2, Histological section of testis of *R. dandia* exposed to 0.10 ppm cadmium during gonadal recrudescence, (H&E; x 400); Spermatozoa (SZ), spermatids (SD); Patches spermatids are prominent than in the control testis.

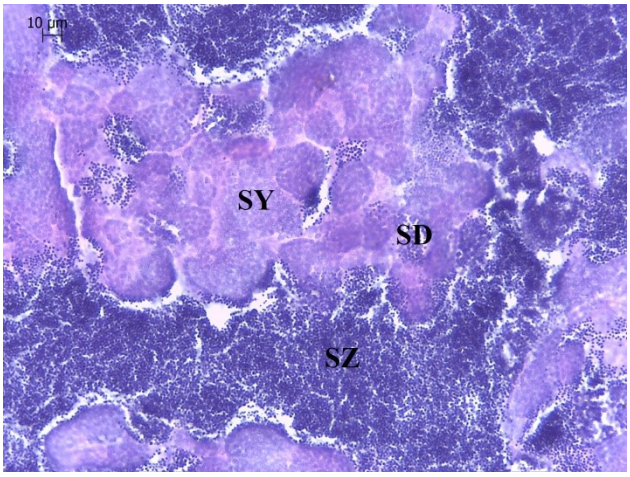


Figure: 5.3, Histological section of testis of *R. dandia* exposed to 0.20 ppm cadmium during gonadal recrudescence, (H&E; x 400); Spermatozoa (SZ), spermatids (SD), spermatocytes (SY); Initial stages of spermatogenesis is prominent.

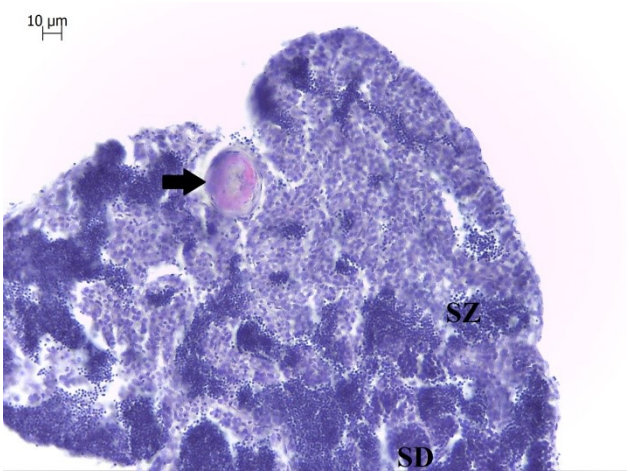


Figure: 5.4, Histological section of testis of *R. dandia* exposed to 0.30 ppm cadmium during gonadal recrudescence, (H&E; x 400); Spermatozoa (SZ), spermatids (SD) Blood vessel (➡); Limited presence of sperm bundles indicating early stage of recrudescence.

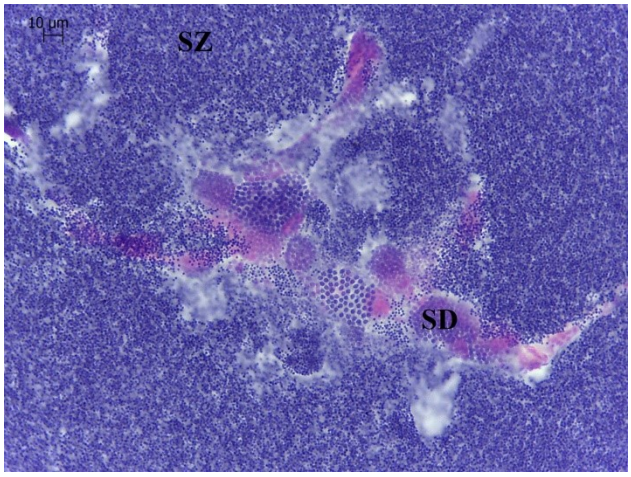


Figure: 5.5, Histological section of testis of *R. dandia* exposed to 1.00 ppb mercury during gonadal recrudescence, (H&E; x 400); Spermatozoa (SZ), spermatids (SD); Spermatids were more abundant than control testis.

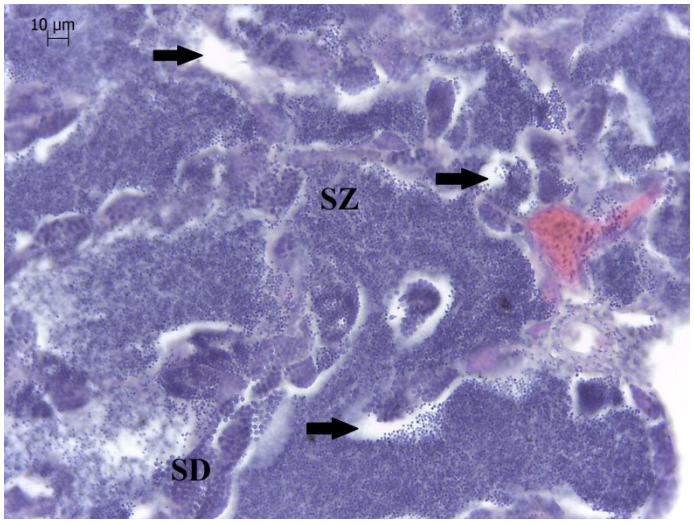


Figure: 5.6, Histological section of testis of *R. dandia* exposed to 2.00 ppb mercury during gonadal recrudescence, (H&E; x 400); Spermatozoa (SZ), spermatids (SD); Vacant areas (➡) present.

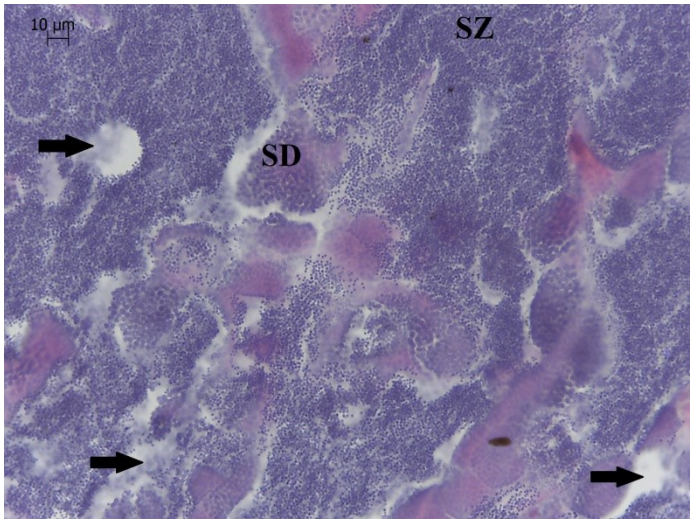


Figure: 5.7, Histological section of testis of *R. dandia* exposed to 3.00 ppb mercury during gonadal recrudescence, (H&E; x 400); Spermatozoa (SZ), spermatids (SD); Vacant areas (➡) present.

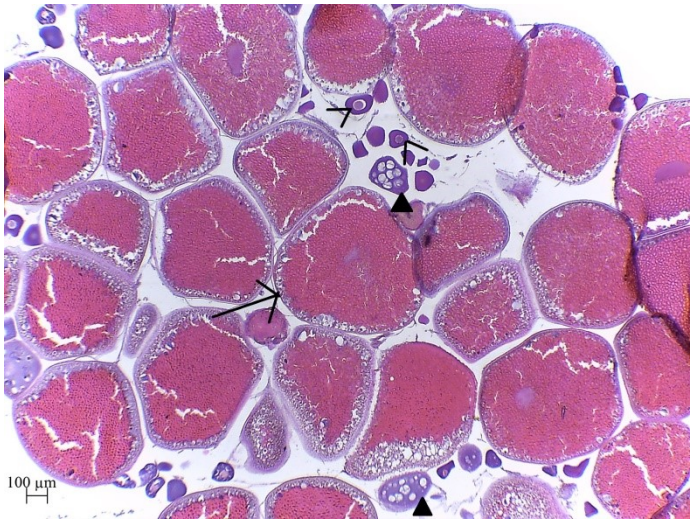


Figure: 5.8, Histological section of ovary of *R. dandia* (Control), (H&E; x 40); Immature oocyte (>), Maturing oocyte (▲), Mature oocyte (→); Ovary with abundant mature oocytes and absence of mass atresia.

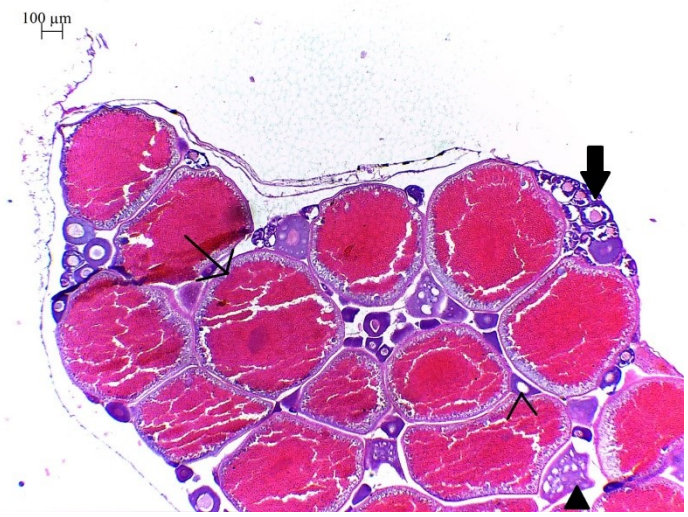


Figure: 5.9, Histological section of ovary of *R. dandia* exposed to 0.10 ppm cadmium during gonadal recrudescence, (H&E; x 40); Immature oocyte (>), Maturing oocyte (▲), Mature oocyte (→); Area of mass atresia of immature oocytes (➡).

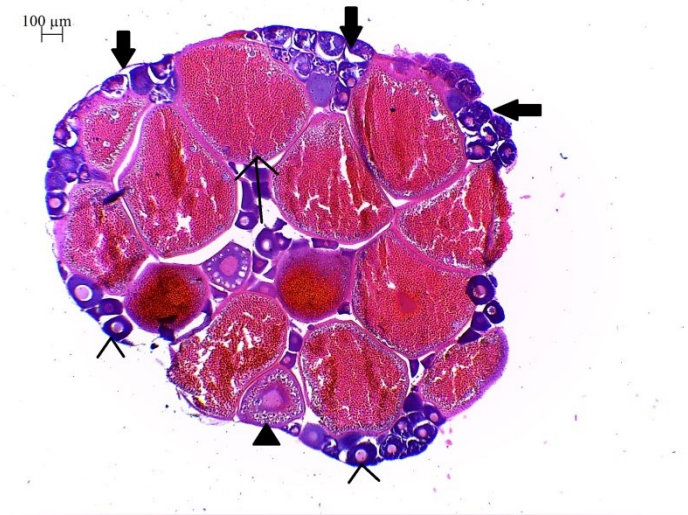


Figure: 5.10, Histological section of ovary of *R. dandia* exposed to 0.20 ppm cadmium during gonadal recrudescence, (H&E; x 40); Immature oocyte (>), Maturing oocyte (▲), Mature oocyte (→); Area of mass atresia of immature oocytes (➡); Mature oocytes are limited.

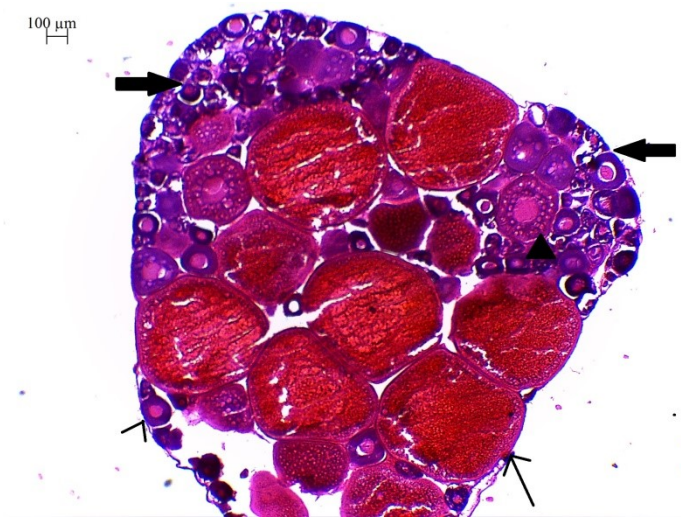


Figure: 5.11, Histological section of ovary of *R. dandia* exposed to 0.30 ppm cadmium during gonadal recrudescence, (H&E; x 40); Immature oocyte (>), Maturing oocyte (▲), Mature oocyte (→); Area of mass atresia of immature oocytes (➡); a few mature oocytes are present.

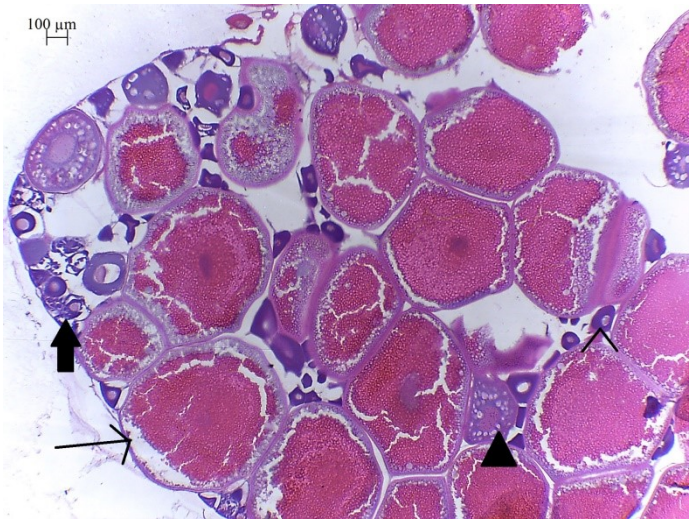


Figure: 5.12, Histological section of ovary of *R. dandia* exposed to 1.00 ppb mercury during gonadal recrudescence, (H&E; x 40); Immature oocyte (>), Maturing oocyte (▲), Mature oocyte (→); Area of mass atresia of immature oocytes (➡).

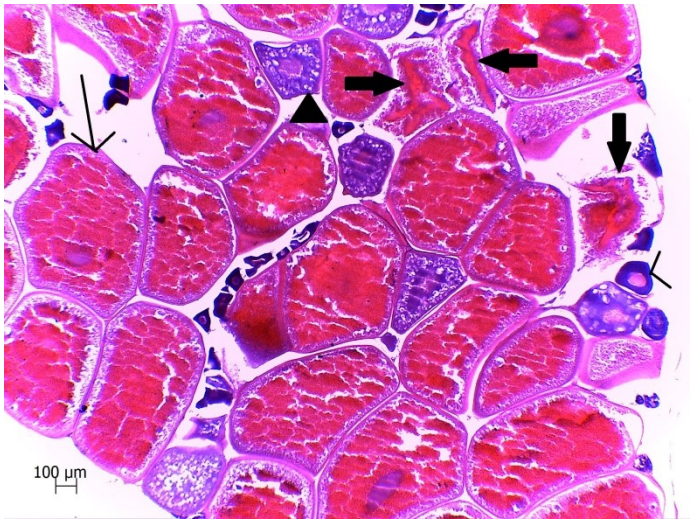


Figure: 5.13, Histological section of ovary of *R. dandia* exposed to 2.00 ppb mercury during gonadal recrudescence, (H&E; x 40); Immature oocyte (>), Maturing oocyte (▲), Mature oocyte (→); Atresia of mature oocyte (➡).

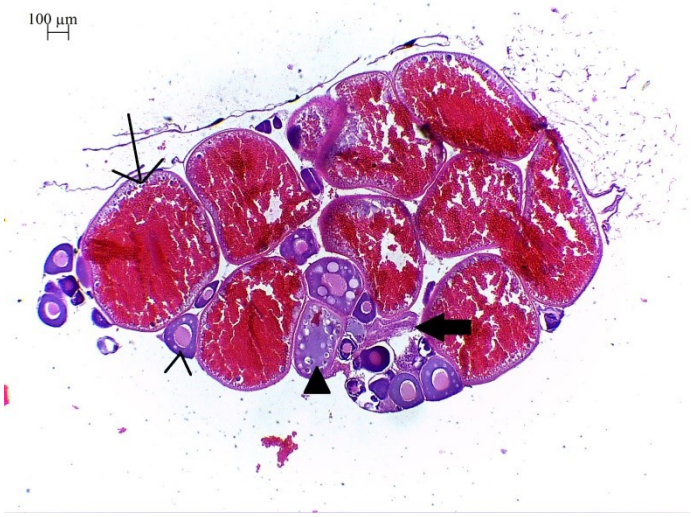


Figure: 5.14, Histological section of ovary of *R. dandia* exposed to 3.00 ppb mercury during gonadal recrudescence, (H&E; x 40); Immature oocyte (>), Maturing oocyte (▲), Mature oocyte (→); Atresia of mature oocyte (➡); Presence of mature oocytes are limited.

Discussion

Gonad maturation is an important phase in the life cycle of fishes as it involves intensive physiological changes leading to spawning with ripe gametes. Environmental toxins like heavy metals have profound influence in altering normal physiological mechanisms. Maintaining homeostasis under toxicant stress is an energetically expensive process demanding high mobilisation of resources (Rajan and Kuzhivelil, 2018). Survival in polluted ecosystems costs reallocation of energy from other physiological processes resulting in diminished growth rate and reproductive potential (Wendelaar Bonga, 1997; Beyers *et al*., 1999 and Marchand *et al*., 2004).

In the present study, GSI was measured as it is a useful endpoint to evaluate reproductive toxicity of chemicals in fishes (Segner, 2011). Robinson *et al*., (2007) observed that the exposure of 669.00 ng L^{-1} of 17 β-oestradiol affected testicular development and reduced GSI in the male sand goby, *Pomatoschistus minutus*. Ram and Sathyanesan (1987) correlated inhibited gonadal growth with reduction in testicular GSI in male *C. punctatus* exposed to organophosphorus pesticide, cythion during gonadal recrudescence. Kirubagaran and Joy (1992) observed reduced GSI in male catfish, *C. batrachus* exposed to mercuric chloride during preparatory phase of annual reproductive cycle. Ram and Sathynesan (1983) observed reduction in GSI of male *C. punctatus* exposed to 0.01 ppm of mercuric chloride during gonadal maturation. In the present study, GSI was found to be declined significantly in male *R. dandia* by increasing concentrations of cadmium exposure during gonadal recrudescence. While reduced GSI of male fishes observed in all mercury exposed groups, significant differences did not occur between various doses used in the present study (table 5.1).

Ram and Sathyanesan (1986) observed significant reduction in GSI of female *C. punctatus*, exposed to mercurial fungicide, Emisan during gonad development phase of annual breeding cycle. Ram and Sathyanesan (1983) exposed female *C. punctatus* to mercuric chloride during gonadal recrudescence and GSI were lower in test fishes than control group at the end of the experiment. Kirubagaran and Joy (1988) studied the effect of mercuric chloride, methyl mercuric chloride and mercuric fungicide, Emisan-6 on the ovarian recrudescence of *C. batrachus.* All the experimental groups were showed significant reduction of GSI compared to control fishes. Ram and Sathyanesan (1987) observed lowered GSI in female *C. punctatus*, under the influence of organophosphorous pesticide, Cythion during gonadal recrudescence. Pereira *et al*., (1993) exposed female winter flounder, *Pleuronectes americanus* to cadmium in seawater for a period of 71 days. Although hepatosomatic index decreased with increasing cadmium concentration, GSI was not affected. In the present study exposure to various concentrations of mercury reduced GSI of female *R. dandia* but a dose dependent influence did not observe. 0.10 ppm and 0.20 ppm cadmium exposure during recrudescence reduced GSI of female fishes without significant differences between two doses while 0.30 ppm cadmium exposure caused further reduction in GSI of female *R. dandia* (table 5.2).

According to Kime (1999) it is often unclear whether decreased GSI under xenobiotic influence is attributed to the direct effect on gonads or the result of deficiency in pituitary hormone secretion. Pituitary gland plays a critical role in fish reproduction through the secretion of gonadotropins which induce steroid hormones synthesis and stimulate gonadal development (Crump and Trudeau, 2009; Zohar *et al*., 2010). Gonadotropin secreting cells of pituitary showed inactivation and accumulation of secretary products in catfish, *C. batrachus* under cadmium exposure

(Jadhao *et al.,* 1994). Apart from the direct influence on gonadal physiology, xenobiotic effects on fish reproduction may be mediated through other components of reproductive system.

Mani and Saxena (1985) studied the effects of carbofuran and fenitrothion on the testicular recrudescence of *C. punctatus*. Both pesticides delayed the formation of spermatids and sperm as well as caused necrosis in the testes. Ram and Sathyanesan (1983) exposed *C. punctatus* to mercuric chloride during gonad recrudescence and observed that the testes of exposed fishes were filled with secondary spermatogonial cells, while control fishes exhibited active spermatogenesis with mature sperm. In the present study, initial stages of spermatogenesis were prominent and abundance of mature sperm was reduced in test fishes compared to control fishes (figures 5.1, 5.3, 5.4 and 5.7). This indicates that cadmium and mercury treated fishes were not progressed in testicular recrudescence as in control fishes.

Ram and Sathyanesan (1987) observed retarded ovarian growth in pesticide cythion treated *C. punctatus* during gonad maturation. Prominence of immature oocytes, reduced presence of maturing oocytes as well as atresia observed in treated fishes. *C. punctatus* exposed to fungicide, Emisan during recrudescence showed predominance of early oocyte stages while majority of oocytes completed vitellogenesis in control fishes (Ram and Sathyanesan, 1986). Exposure of *C. batrachus* to mercuric chloride, methyl mercuric chloride and emisan-6, arrested ovarian recrudescence and oocytes were in non vitellogenic stage (Kirubagaran and Joy, 1988). In the present study, fishes exposed to 3.00 ppb mercury, 0.20 ppm and 0.30 ppm cadmium during gonadal recrudescence had impaired gonad maturation as evidenced by limited presence of mature oocytes (figures 5.10, 5.11 and 5.14), while mature oocytes were abundant in the ovaries of control fishes (figure 5.8). Presence of

mass atresia indicates ovarian degeneration in heavy metal exposed fishes (figures 5.9, 5.10, 5.11 and 5.12). Although atresia of immature oocytes was common in all experimental groups of fishes, atresia of mature oocytes was evident only under 2.00 ppb and 3.00 ppb mercury exposure (figures 5.13 and 5.14).

Pollutants which affect vitellogenin synthesis can hinder ovarian recrudescence of fishes. Vitellogenin is the chief precursor of egg yolk protein produced by the liver of female fishes which is released into the blood and utilised in oogenesis (Hara *et al*., 2016). Synthesis of vitellogenin precursors by liver is induced by estrogen in fishes (Reading and Sullivan, 2011). High levels of accumulation of heavy metals in the ovaries are linked to reduction of estradiol in female fishes in highly polluted areas (Ebrahimi and Taherianfard, 2010). Dietary exposure to methyl mercury significantly reduced 17β-estradiol and inhibited gonad development in female fathead minnows, *P. promelas* (Drevnick and Sandheinrich, 2003). Zinc, lead and mercuric acetate injection were found to decrease serum vitellogenin level in *C. batrachus* (Panigrahi *et al*., 1990).

Cadmium was found to cause reproductive endocrine disruption in fishes (Thomas, 1993; Mukherjee *et al.,* 1994; Amutha and Subramanian, 2013; Kim *et al.,* 2016). Release of gonadal steroids was significantly reduced following exposure to cadmium in Japanese medaka (Tilton *et al.,* 2003). Ma *et al.,* (1995) observed significant lowering of serum gonadotropin on cadmium exposure in common carp, *C. carpio*. Variation in steroid production disrupts feedback pathways which orchestrate hypothalamus–pituitary–gonadal axis, ultimately leading to reproductive impairment (Arcand-Hoy and Benson, 1998). Further studies are required in *R. dandia* to prove that how the cadmium and mercury affects the reproductive physiology of the fish.

Dietary exposure of cadmium caused reduction in the number of spermatozoa and milt volume in red tilapia (El-Ebiary *et al.,* 2013). Reduction in ripe spermatozoa under the influence of water borne cadmium and mercury was evident from the present study (figures 5.1, 5.3, 5.6 and 5.7). Most commonly found strategy in teleost fishes is external fertilization in which gametes are released into the external environment and sperm activity decline in a brief period of time (Coward *et al.,* 2002). Ciereszko and Dabrowski, (1994) observed that motility, sperm concentration and fertilisation were strongly correlated in rainbow trout. Reduced number of spermatozoa as observed in the present study reduces chances of fertilisation in the external environment which may result in declined number of progenies.

In the present study, lower concentrations of heavy metals did not entirely arrest gamete maturation (figures 5.2, 5.5, 5.9 and 5.12). But impact on reproductive potential is indicated by alterations like lowered GSI, reduced abundance of spermatozoa and mature oocytes and increased oocyte atresia in test fishes (tables 5.1and 5.2; figures 5.4, 5.9 and 5.14). According to Kime and Nash (1999), even though lower levels of pollutants are incapable of hindering gametogenesis, they may cause gamete abnormalities by disrupting optimal endocrine balance. Sperm flagellar length was shortened causing significant decrease in motility occurred in gold fish sperm after exposure to 0.10 mg L^{-1} mercuric chloride (Van Look and Kime, 2003). Abnormalities in gametes may result in anomalies in fish fries developed from them. El-Ebiary *et al.,* (2013) observed decrease in number of spawned eggs, hatchability percentage and increased fry abnormalities under the influence cadmium in red tilapia although gonad development was unaffected under same doses of cadmium.

Seasonal breeding in fishes is a strategy to synchronize reproductive efforts to periods ideal for juvenile survival and growth (Winemiller, 1993). Delayed gonad

maturation under toxicant stress may prevent utilisation of optimal climatic conditions for spawning and larval growth. Reduced chance of larval survival in unfavorable environmental conditions causes reduction in the number of progenies. Toxicant stress during gonad maturation results in lowered reproductive potential in fishes as lower number of spermatozoa can lead to severe impact on population size. Thus, toxic effects in gonadal recrudescence may pose direct threat to the wild fish populations eventually leading to decline in species diversity. Being a sensitive endpoint, gonad maturation dysfunction in fishes offers prompt indicators of toxicant stress in ecological monitoring programs.

CHAPTER 6

EFFECTS OF HEAVY METALS ON THE EMBRYONIC DEVELOPMENT

Introduction

Reproduction is an essential physiological process which ensures the continuity of the species. Investigating the effect on reproductive success of organisms is essential in assessing long term impact of a pollutant in an ecosystem. Vulnerability of population to toxicant stress can be assessed comprehensively by studying impacts on various life stages (Luckenbach *et al*., 2001). According to Jezierska *et al*. (2008), larval stages are particularly sensitive to pollution. Embryonic development is especially prone to detrimental effects of toxicants in water in the case of aquatic organisms like fishes, which release gametes into external environment. Early ontogenic stages are ideal for testing toxicity as they are more sensitive to toxicant stress than juvenile and adult fishes (Dave and Xiu, 1991; Hutchinson *et al.,* 1998; Foekema *et al.,* 2008 and Mhadhbi *et al.,* 2010).

Along with the detrimental effects on gonads, heavy metals are also identified as developmental toxins inducing teratogenic effects in animals (MacRae and Pandey, 1991; Soukupova and Dostal, 1991; Domigo, 1994; Tchounwou *et al*., 2012). Embryonic development occurs in the external environment among many fishes, making them susceptible to developmental toxicity of metals in the surrounding water. Apart from the direct influence on developing stages of life cycle, waterborne metals reduce gamete quality and viability by accumulating in the gonads (Jezierska *et al*., 2008). Early life stages are more vulnerable to toxic effects of xenobitotics than the adults due to the metabolic peculiarities of growth and development (Oskarsson *et al*., 1998; Vasconcelos *et al*., 2010; Staples *et al*., 2011). Early life stages may lack enzymatic mechanisms of detoxification, making them susceptible to toxins which do not influence adult organisms (Vasconcelos *et al*., 2010). Heavy metals are associated

with fish deformities which in turn have devastating effects on fish populations (Sfakianakis *et al*., 2015).

Even though fish embryos are protected by the egg shell, they are under direct contact with the external aquatic environment which may contain an array of toxicants. According to Jezierska *et al*., (2008) embryos are not provided full protection from metal diffusion especially during the initial phases of swelling through absorption of water by the perivitelline space. The course of embryonic development is a fragile period in the life cycle of an organism. The complex processes which convert a zygote into a multicellular organism are often influenced by toxins at concentrations even lower than that required to impart general cellular toxicity (Bantle, 1995). Compared to adult and juvenile fishes, embryonic stages are more sensitive to toxicants (Dave and Xiu, 1991; Sloman and McNeil, 2012).

Osman *et al*., (2007) investigated the impact of lead nitrate exposure on the embryos of *C. gariepinus*, an African catfish. Morphological anomalies like yolk sac oedema, pericardial oedema, notochordal defect and irregular head shape were observed. Anomalies increased in frequency with the increase in lead concentration given to embryos. Hatching time was delayed and frequency of hatching success declined with lead exposure. Cai *et al*., (2012) exposed zebra fish embryos to a range of cobalt concentrations. The early development and survival of embryos were not significantly affected at concentrations less than 100 µg/L. But at higher concentrations, survival rates are reduced and developmental anomalies like abnormal morphology, retarded growth, bradycardia and delayed hatching were observed. Johnson *et al*., (2007) studied the effect of copper on the development of zebra fish,

D. rerio. Survival of the zebra fish embryos was considerably declined and enlarged yolk sac with decreased body length at 72 hour post fertilization was observed.

Witeska and Lugowska (2004) studied the influence of copper exposure during embryonic development in common carp. Copper exposure during embryonic development caused reduced hatching rate and elevated body malformations in newly hatched larvae. Shortened body, C-shaped larvae, deformed yolk sac and curvature of the spine were observed among malformed larvae. Witeska *et al*., (2014) studied the effects of cadmium and copper in the development of European cyprinid fish, *Leuciscus idus*. Embryonic survival was reduced significantly and increased frequency of body malformations and death was found in newly hatched larvae among heavy metal exposed groups. Delayed development was observed in the onset of active feeding, swim bladder inflation and yolk utilization. Ismail and Yusof (2011) exposed newly fertilized eggs of *Oryzias javanicus*, to various concentrations of mercury and cadmium. Several developmental abnormalities leading to mortality are observed under exposure levels ranging from 0.01 ppm to 0.05 ppm of mercury and cadmium. Immediate arrest of development was observed in exposure to 0.10 ppm cadmium. Embryos were more sensitive to cadmium exposure than mercury exposure (Ismail and Yusof, 2011).

Zhu *et al*., (2014) studied the developmental toxicity of heavy metals, zinc, cadmium and copper in the embryos of rare minnow, *Gobiocypris rarus*. Most notable anomalies are body and heart malformations and a dose response relationship is provided by increased malformations. Dave and Xiu (1991) investigated the toxicity of nickel, copper, lead, mercury and cobalt in the larvae and embryos of zebra fish, *Brachydanio rerio.* Nickel and copper were found to be the specific hatching

inhibitors compared to lead, mercury and cobalt. Weis and Weis (1977) exposed *Fundulus heteroclitus* embryos to various concentrations of heavy metals. Cyclopia were observed in inorganic mercury treated embryos whereas lordosis observed in lead exposure. Cadmium did not induce considerable effects up to 10 ppm on *F. heteroclitus* embryos.

The previous studies showed that, apart from reduction in survival rates, heavy metals exposure imparts various structural malformations in fish embryos. Reduced larval survival impacts population size whereas larval anomalies lead to reduction in healthy individuals with persistence in adverse conditions as well as reproductive potential. This implies the deteriorating influence of heavy metal pollution in wild fish populations through developmental toxicity, apart from their vicious influence in the mortality and health of adult individuals. Impact on wild fish populations is in turn detrimental to other piscivorous species in the food chain leading to instability in the ecosystem. Dwindling of fish stocks are also disadvantageous to human societies as they form an important source of food.

Many studies on embryonic toxicity of metals are focused in culture fishes (Dave and Xiu, 1991; Witeska and Lugowska, 2004; Johnson *et al*., 2007; Osman *et al*., 2007; Cai *et al*., 2012). Extending developmental toxicity studies to various wild fish species aids in obtaining much detailed picture of ecological impact of heavy metals. It also evaluates the sensitivity and resilience of various taxonomic groups to toxic stress. Here, the influence of two heavy metals, mercury and cadmium on the embryonic development of native fish *R. dandia* is investigated.

Materials and methods

During monsoon season, the teleost fish *R. dandia* were collected, transported, acclimatized and maintained in the laboratory as described in chapter 2 of the thesis. Sexually mature, gravid healthy fishes were selected and induced breeding was carried out as described in chapter 2. The fertilized eggs were collected carefully and gently as described in chapter 2 and transferred to Petri dishes. The Petri dishes are filled half with water with physico chemical features described in chapter 2. The Petri dishes were gently aerated.

Five experimental groups were created with three replicates. Each experimental group consist of 50-60 fertilized eggs on an average in each Petri dish. The groups were exposed to 50 ppb and 100 ppb of mercury (mercuric chloride as source, Loba Chemie, India, extra pure grade) and cadmium (cadmium chloride as the source, Merck India, analytical grade) while one group is set apart without metal as control. The water was changed every 24 hours by gentle siphoning and replaced with fresh water or fresh water containing respective amount of metal. During embryonic development, observations were made for hatching of the larvae twice a day and results are recorded on 24 hours basis. The hatched larvae were counted and percentage of total hatching was calculated to determine hatching success. Beginning with 72 hours, the developing larvae were provided with *Artemia* nauplii on daily basis. At the end of the 120 hours, number of larvae with abnormal morphology were counted under the light microscope (Leica Microsystems, Germany) connected to a computer and abnormal morphology was studied. The morphologically abnormal larvae were photographed using a stereoscopic microscope (Leica M 205 C, Germany) with the help of the software Leica application suite (LAS) version 4.3.0.

The control larvae were also observed under the light microscope and studied for any structural abnormalities. After taking photographs all the larvae were returned to the aquaria. (Witeska *et al*., 2014).

Significance of hatching success and rate of abnormal morphology was analysed using one way analysis of variance (ANOVA) followed by Tukey's test using SPSS 16.0 software.

Results

Mercury exposed larvae exhibited various structural abnormalities comparing to control larvae. Most of the mercury exposed larvae have stouter appearance with reduced body length ((figures 6.3, 6.4, 6.5 and 6.6). Mercury exposed larvae showed enlarged yolk sac compared to controls (figures 6.2 and 6.3). Along with the enlarged yolk, some larvae exhibited cardiac oedema. Eyes were small and rudimentary in most of the larvae (figure 6.3). Swim bladder inflated in control larva before 96 hours. Mercury exposed larvae didn't show any signs of swim bladder inflation even after this period. Fin folds showed crinkled appearance in caudal region, where it showed smooth appearance in control larvae (figure 6.2 and 6.6). Various vertebral anomalies appeared in mercury exposed larvae. Axial spine curvature – lordosis is frequent among them (figure 6.4). Axial curvature in caudal region also appeared in mercury exposed larvae (figure 6.5).

Compared to control larvae, cadmium exposed larvae showed oedematous structures on cephalic and trunk regions typically (figure 6.8). Swim bladder is not inflated at all or reduced in size among cadmium exposed larvae. Failure to straighten up resulting in c –shaped appearance was a common feature in experimental larvae (figure 6.7). Kyphosis *i.e*., exaggerated rounding of the back occurred among

cadmium exposed embryos (figure 6.9). Scoliosis and lordosis also appeared, but less frequently.

During the beginning of hatching, hatching rate is elevated among mercury and cadmium exposed larvae (Table 6.1). Most of the larvae hatched with abnormal morphology when exposed to mercury and cadmium, whereas abnormal morphology is very rare among control group (Figure 6.1).

Table 6.1: Effect of heavy metal exposure in the percentage of hatching rate of larvae (Mean ± SD). Results are the mean of three replicates of each group. Values in the same column with different superscript letters vary significantly between exposure groups ($p<0.05$; One way-ANOVA, Tukey's post-hoc test).

	24 hours	48 hours	72 hours	96 hours	120 hours
Control	0	18.96 ± 3.43^{a}	79.18 ± 7.96^{a}	84.27 ± 4.80^{a}	88.72 ± 3.41^{a}
50 ppb of cadmium	0	36.67 ± 5.77^{b}	83.33 ± 2.89^{a}	86.67 ± 5.77^{a}	88.33 ± 2.89^{a}
100 ppb of cadmium	0	38.69 ± 5.09^{b}	78.73 ± 4.71^{a}	85.54 ± 6.31^{a}	86.99 ± 3.80^{a}
50 ppb of mercury	0	35.46 ± 1.91^{b}	62.9 ± 6.52^{b}	82.84 ± 2.62^{a}	88.19 ± 3.88^{a}
100 ppb of mercury	0	31.31 ± 3.5^{b}	58.77 ± 4.00^{b}	65.53 ± 5.95^{b}	75.45 ± 1.65^{b}

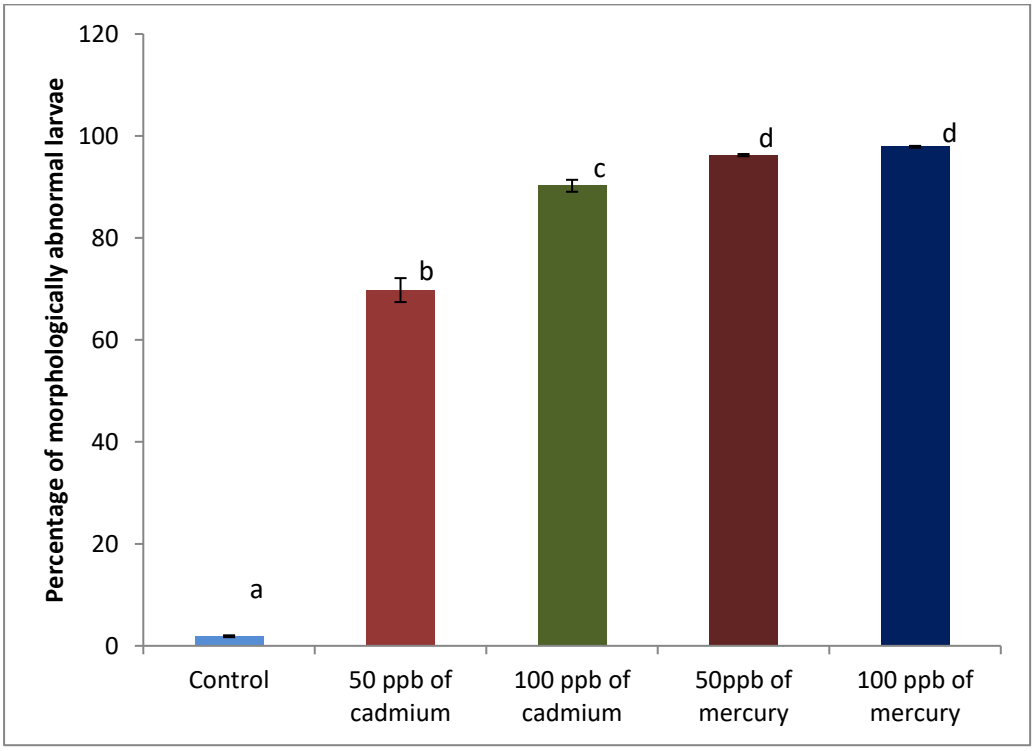


Figure: 6.1, Effect of heavy metal exposure in the percentage of morphologically abnormal larvae (Mean ± S.D). Results are the mean of three replicates of each group. Different superscript letters indicate significant variation between exposure groups ((p<0.05; One way-ANOVA, Tukey's post-hoc test).

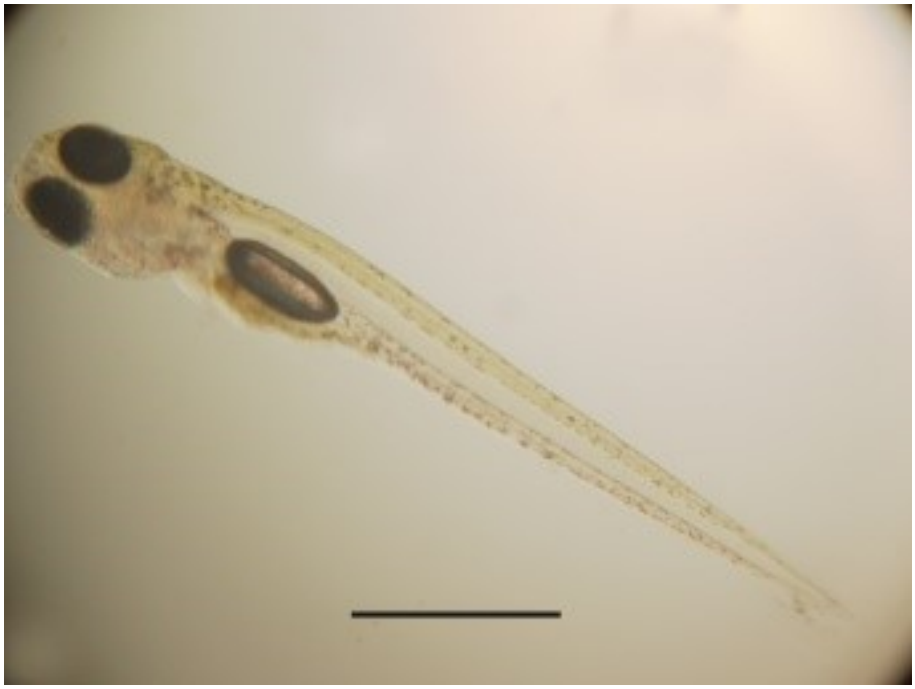


Figure: 6.2, Control larva at 120 hours of development; Scale bar -1 mm.

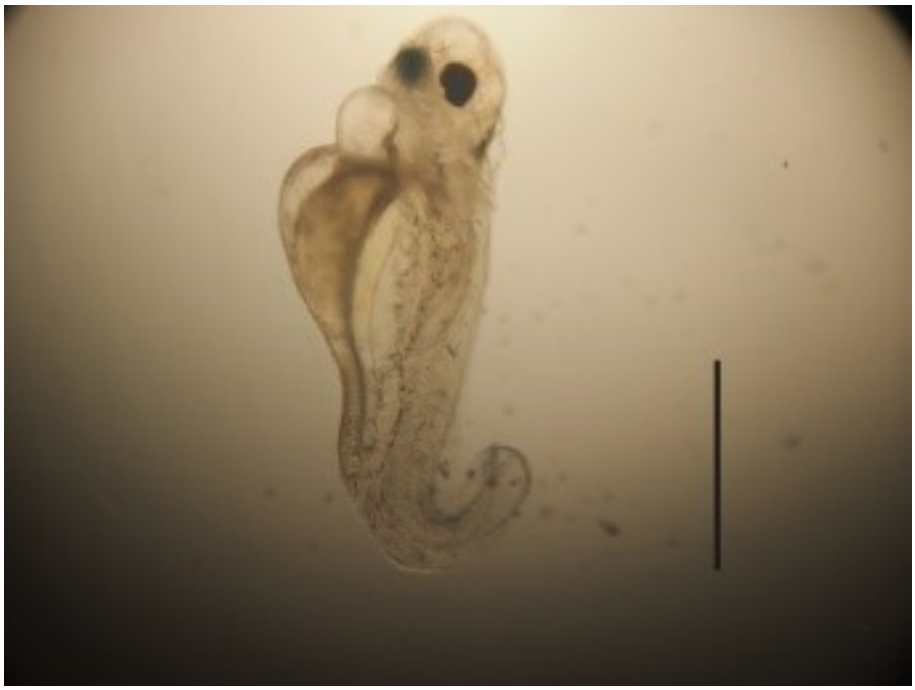


Figure: 6.3, 100 ppb mercury exposed larva at 120 hours of development with yolk sac oedema, cardiac oedema and rudimentary eyes; Scale bar -1 mm.

Figure: 6.4, 100 ppb mercury exposed larva at 120 hours of development with yolk sac oedema, cardiac oedema and lordosis; Scale bar -1 mm.

Figure: 6.5, 100 ppb mercury exposed larva at 120 hours of development with yolk sac oedema, cardiac oedema and axial curvature in caudal region; Scale bar -1 mm.

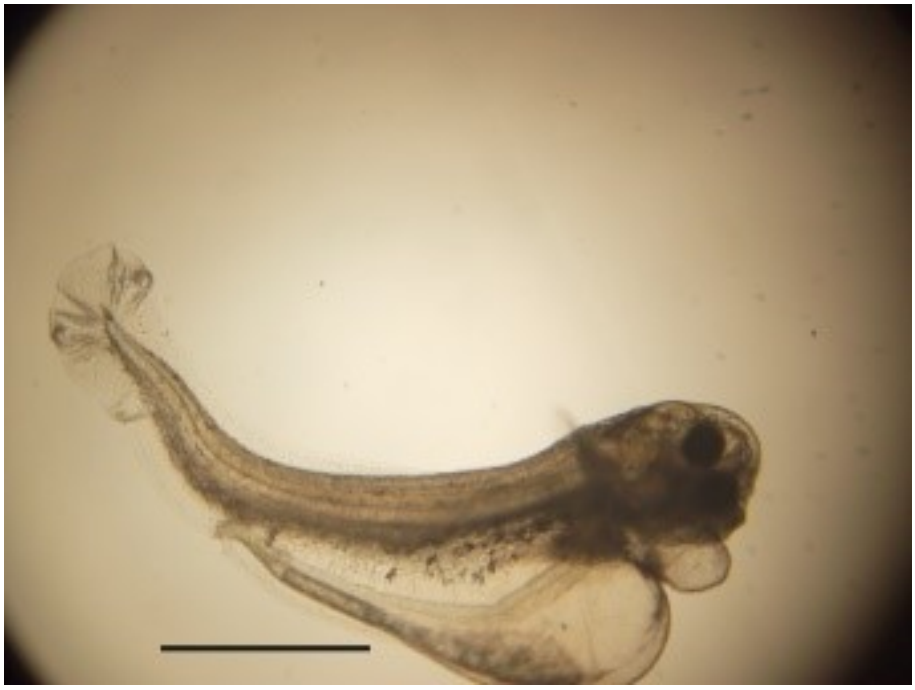


Figure: 6.6, 50 ppb mercury exposed larva at 120 hours of development with yolk sac oedema, cardiac oedema and crinkled fin folds in caudal region; Scale bar -1 mm.

Figure: 6.7, 100 ppb cadmium exposed larva at 120 hours of development with c shaped structure; Scale bar -1 mm.

Figure: 6.8, 100 ppb cadmium exposed larva at 120 hours of development with oedematous structures on cephalic and trunk regions; Scale bar -1 mm.

Figure: 6.9, 100 ppb cadmium exposed larva at 120 hours of development with kyphosis; Scale bar -1 mm.

DISCUSSION

The course of embryonic development is a fragile period in the life cycle of an organism. Responses required to overcome early environmental influences disrupting normal development may have long time consequences in the life of the adult organism (Gluckman *et al*., 2005). Several studies have documented viciousness of heavy metals as toxins which interfere in normal development of various groups of animals (Ravera, 1991; Schmidt, *et al.,* 1991; Gomot, 1998; Kobayashi and Okamura, 2004; Gopalakrishnan *et al.,* 2007 and Johnson *et al.,* 2007; Tchounwou *et al*., 2012; Jaishankar *et al*., 2014). Heavy metals, being one of the major aquatic pollutants are extremely significant as developmental toxins raising a threat to the normal development of fishes.

Although various life cycle stages of fishes are widely used for toxicity tests, some developmental stages are more prone to detrimental effects of toxicants. The embryonic, larval and early juvenile stages have elevated sensitivity to deteriorated environmental conditions. Devising toxicity assays using premature life stages of fishes will be valuable in evaluating safety of wide range of chemicals (Walker *et al.,* 1991; Nguyen and Janssen; 2001 and Fraysse *et al.,* 2006; Villeneuve *et al*., 2014). Thus, tests using these initial life cycle stages provide most factual concepts regarding maximum acceptable limits of toxicants.

In the present study, occurrence of abnormalities in fish larvae exhibited a dose dependent relation to the amount of mercury and cadmium in the water (figure 6.1). The similar trend was previously observed on fish larval abnormalities with heavy metals like copper, zinc, cadmium and lead (Osman *et al.,* 2007; Zhu *et al.,* 2014). Hatching begins after 24 hours of fertilisation and it is observed that mercury

and cadmium treated groups have elevated hatching percentage at 48 hours (table 6.1). At 72 hours hatching rate is found to be decreased among mercury treated groups compared to control group. At 96 hours and 120 hours only 100 ppb mercury treated group exhibited reduced hatching rate (table 6.1). According to Jezierska *et al.,* (2008) various processes of fish embryonic development is influenced by metals to affect the rate of development. Along with deformations, heavy metals can bring about alterations in fish embryonic development, like alteration in the hatching rate.

In the present study, among the mercury exposed larvae, enlarged yolk sac was very prevalent (figures 6.3, 6.4, 6.5 and 6.6), which may be the result of yolk sac oedema or decreased yolk resorption and utilisation during embryonic life. Mercury exposed larvae mostly have rudimentary eyes (figure 6.3), which is a feature does not appear in cadmium treated groups. This deformation may be attributed to known neurotoxic properties of mercury. Vertebral anomalies appeared in all experimental groups of larvae (figures 6.4, 6.5, 6.7 and 6.9). The present study shows that water borne heavy metals can interfere in organogenesis, resulting in various malformations in the fish larvae (figures 6.3, 6.4, 6.5, 6.6, 6.7, 6.8 and 6.9). All this will result in poor quality of progeny which are less efficient in survival and other important life cycle processes like reproduction.

Environmental conditions in the developing stages affect well-being of the organism throughout the adult life. Although some individuals survive adverse environmental influences during development, they come at the cost of bearing detrimental effects with long term consequences in the adult life (Gluckman *et al*., 2005). Establishing a stable population by a fish species requires proper environmental situations facilitating uninterrupted development enabling successful

recruitment (Luckenbach *et al*., 2001). Teratogenic impacts of toxicants leading to sterility in adult life indirectly contribute to dwindled population size in the future. Malformations induced by toxins may contribute to individuals which are less fit to avoid predation, again contributing to the decreased population size.

Early life stages are not very accessible in traditional mammalian toxicology models (Yang *et al.,* 2009). Experimental accessibility in the early development of fishes is advantageous over mammalian models (Bardet *et al*., 2006). Features like availability of large broods, transparency, development outside the maternal body and easy maintenance increase the value of fish embryos as experimental models (Hill *et al*., 2005; Braunbeck, 2006; Yang *et al.,* 2009). Alternative animal models are a necessity to reduce limitations arising in toxicological studies from ethical issues and constrains of resources in traditional mammalian models (Peterson *et al*., 2008). Mammals are indispensable in testing chemicals for human application, yet the usage of alternative systems like fish embryos in early stages aids in using fewer mammals for testing and cut down the expenses (Peterson *et al*., 2008; Yang *et al.,* 2009).

The fish embryo toxicity tests also offer an appropriate substitute option for toxicity testing using adult fishes (Embrya *et al*., 2010; Belanger *et al.,* 2013; Sehonova *et al*., 2015). Fish embryos provide good models for investigating toxic mechanisms and suggest the possibility of long term adverse effects, apart from their utility in acute toxicity tests (Scholz *et al*., 2008). Early life cycle aquatic organisms are the best options for toxicity assays in establishing tolerable limits of contaminants and toxicants in water. They are more convenient, economic and effective toxicity testing models compared to adult animals.

CHAPTER 8

RECOMMENDATIONS

Aquatic environments are under the constant threat of toxicants from various anthropogenic sources. Heavy metals are among the major pollutants raising serious problems to the balance of aquatic ecosystem. Mercury and cadmium are two highly toxic heavy metals with no known biological role. Fishes are significant part of almost all aquatic biota and this taxonomic group is which suffer a major threat from the impact of pollutant contaminants in their environment. Besides being a significant food source of humans, fishes are also serving an important role as model organisms in biological investigations. Reproduction is an essential biological process which ensures the continuity of the species. *R. dandia* is selected as experimental species to investigate the effect of mercury and cadmium due to its availability and sturdiness in laboratory conditions. The effect of mercury and cadmium on the reproduction of *R. dandia* is carried out in the present study.

Relevant findings of the present study can be summarised as:

- Phylogeny analysis using 658 nucleotide length of DNA sequence from the mitochondrial cytochrome C oxidase subunit I (COI) gene provides molecular evidence to taxonomic identity of the species *R. dandia*.

- *R. dandia* is a seasonally breeding fish with a prolonged breeding season extending from June to November. Sexual dimorphism is not conspicuous in the fish. Five maturity stages were identified in the gonads during the reproductive cycle of the fish: immature, maturing, mature, ripe and spent. Induced breeding of *R. dandia* was found successful with HCG at a dosage of 5000 IU (male) and 10,000 IU (female) per kg body weight of fish.

- 96 hours LC_{50} concentration of *R. dandia* for mercury and cadmium was found to be 133.3 ppb and 16.91ppm respectively.

- Bioaccumulation study showed the absence of cadmium and mercury in control samples, while test fishes exhibited accumulation of these heavy metals in the gonads. Accumulation of cadmium increased as exposure period proceeds and both testes and ovaries exhibited this pattern. Mercury progressively accumulated in the testes and ovaries up to 20 days of exposure period. While ovary exhibited increased mercury accumulation at 30 days, testes showed no further increase in accumulation from the 20th day.

- GSI of sexually mature *R. dandia* was found to be affected by mercury and cadmium exposure. Reduced spermatozoa, proliferation of interstitial tissue, disorganised bundles and appearance of vacant spaces were the major histopathological changes occurred in the mature testes of *R. dandia* under mercury exposure. Dilated blood vessels, reduction in spermatozoa and detachment of basement membrane were the significant histopathological changes occurred in the mature testes of *R. dandia* under cadmium exposure. Mass atresia occurred in the mature ovary of *R. dandia* under mercury exposure. Further, fibrotic tissue appeared in the areas where oocytes disappeared. Ripe ovaries transformed into shrunken immature ovaries with histopathological alterations under mercury exposure. Appearance of areas with mass cellular atresia was the main histopathological alteration occurred in the mature ovary of *R. dandia* under cadmium exposure.

- Mercury and cadmium exposure affected gonadal recrudescence of *R. dandia*. At the end of experimental period which conducted during gonad development season, gonads of control fishes became sexually mature with abundant ripe spermatozoa. Ripe spermatozoa were limited and early stages of

spermatogenesis were prominent in cadmium treated male fishes. Less dense spermatozoa with large patches of spermatids were found in the testis of mercury exposed fishes signifying the impact of this toxicant on gonad maturation. Female control fishes showed sexually mature ovary with abundance of mature oocytes. Mass atresia of immature oocytes occurred in the ovary of cadmium treated fishes. Mature oocytes were reduced in cadmium exposed fishes. Atresia of mature oocyte also occurred along with the mass atresia formed in the immature oocytes of fishes exposed to mercury. Heavy metal treated fishes showed lowered GSI compared to control fishes at the end of exposure period.

- Heavy metal exposure during the embryonic development induced various structural anomalies in the larvae of *R. dandia*. Vertebral abnormalities were common in all experimental groups of larvae. Swim bladder was found to be inflated in control larva before 96 hours. Mercury exposed larvae didn't show any signs of swim bladder inflation even after this period. Swim bladder was not inflated at all or reduced in size among cadmium exposed larvae. Rudimentary eyes and enlarged yolk sac were prevalent among mercury exposed larvae. C-shaped larvae were common among cadmium exposed group. Mercury and cadmium treated larvae have elevated hatching percentage at 48 hours. Hatching rate was found to be decreased among mercury treated groups compared to control group at 72 hours. Only 100 ppb mercury treated group exhibited reduced hatching rate at 96 hours and 120 hours.

The present study indicates that the reproductive phases of the fishes are vulnerable to mercury and cadmium toxicity. Impact of these heavy metals on the

embryonic development indicates the formation of poor quality of progeny which may end up in reduced population size. The study indicates heavy metals may impair reproduction in fishes and this may lead to the decline in fish populations. Unless checked effectively, heavy metal pollution will cause serious impact on wild fish populations. This may in turn disturb the overall balance of aquatic ecosystems. Also, reproductive toxicity parameters may be effectively developed into biomarkers of toxicant contamination in ecosystem monitoring programmes.

CHAPTER 7

SUMMARY AND CONCLUSIONS

Reproduction among fishes is a vital physiological function that maintains continuity of the race. From the ecotoxicological point of view, reproduction is an important end point too. Reproduction in wild fishes is further to be explored as the knowledge about the reproductive mechanism is important in fish population studies and stock assessment.

Since aquatic pollution from anthropogenic sources are increasing, biomonitoring of water samples becomes essential for understanding the level of the contaminant. The chemical analysis of water samples is to be supplanted with biological data. Batteries of biomarkers, including histopathological biomarkers of stress, which can be developed from reproductive organs of fishes can serve this duty. The present study shows that, the parameters from fish reproductive organs under heavy metal stress, like dilated blood vessels, reduction in the number of spermatozoa, detachment of basement membrane of testis and mass atresia, appearance of fibrotic tissue in ovary, all can be suggested as suitable candidates for histopathological biomarkers of heavy metal stress.

Further, lowered gonadosomatic index (GSI), vertebral abnormalities during embryonic development of teleost fishes under heavy metal stress are also parameters that can be considered as suitable biomarkers of heavy metal stress. However, present laboratory experiments require validations from field studies for the use of these biomarkers for biomonitoring programmes.

All stages of teleost fish reproductive process are found to be highly vulnerable to the stress of heavy metals. As the heavy metals can impair reproduction in wild animals like fishes, pollution in general and heavy metal pollution from anthropogenic sources in particular is to be reduced in ecosystems.

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