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Mercury Traced to the Tissue and Cells of the Testes of the Marine Teleostean Fish *Leiostomus xanthurus*, and Mercury-induced Testicular Histopathology.

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Abstract

Fishes take up Hg from the environment directly in its inorganic (HgII) form, and from their food primarily as methylated organic (MeHg). The effects of HgII and MeHg on fish reproduction are well documented for ovaries, but less so for testes. Auto metallography visualized Hg-sulfide granules in testes. Whether assimilated from their environment, or amplified by feeding on Hg-contaminated food, identifiable Hg granules were evident in spermatogenic germ cells, epithelial cells, and connective tissue surrounding sperm ducts, Sertoli cells of the walls of spermatocytes, and spermatozoa. Tissue and cellular locations of granules coincided with areas where Histopathological effects are evident in other fishes. These effects compromise testicular function.

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Introduction

Mercury (Hg), deposited from the atmosphere into marine and freshwater environments in its inorganic (HgII) form, biomagnifies through food webs in its methylated organic (MeHg) form (Driscoll., *et al*. 2013). Both HgII and MeHg are toxic to aquatic and marine invertebrates and fish (Kasper., *et al*. 2009; Liu., *et al*. 2012), terrestrial vertebrates, and humans (Sweet and Zelikoff, 2001).

Both HgII and MeHg, absorbed from ingested food in the alimentary canal, have particular pathological effects on the reproduction of fishes (Crump and Trudeau, 2009). Effects are well documented for ovaries (Govoni., *et al*. 2017); less comprehensively for testes. In ovaries, dietary exposure to HgII and MeHg results in histopathological effects, including apoptosis of follicles and increases in atretic oocytes, along with compromised ovarian production, registered as decreased egg production, decreased egg size, and decreased hatch rate (Govoni., *et al*. 2017). In testes, Hg exposure results in histopathological effects that culminate in compromised spermatogenesis; reduced sperm production and abnormal or necrotic spermatozoa (Table 1). Batchelar., *et al*. (2013) found an association between the proportion of primary spermatocytes and total Hg concentration in muscle in fish caught in a heavily contaminated lake.

Histopathological effect	Reference
Tissue level	
Suppressed testicular development or suppressed spermatogenesis	Ram and Sathyanesan (1983); Wester (1991); Kirubagaran and Joy (1992); Friedmann., *et al*. (1996); Vergílio., *et al*. (2014); Aziz., *et al*. (2017)
Testicular degeneration, hypertrophy, or atrophy	Wester and Canton (1992); Friedmann., *et al*. (1996): Miles-Richardson., *et al* (1999); Weis (2009); Ebrahimi and Taherian-fard (2009); Rajan and Kuzhivelil (2013); Vergílio., *et al*. (2014)
Disorganization of testicular tissue or disorganization of cyst structure	Wester and Canton (1992); Hammerschmidt., *et al*. (2002); Liao., *et al*. (2006); Ebrahimi and Taherianfard (2009); Vergílio., *et al*. (2014); Aziz., *et al*. (2017)
Congestion in testicular circulatory vessels	Vergílio., *et al*. (2014)
Proliferation of interstitial tissue	Rajan and Kuzhivelil (2013); Vergílio., *et al*. (2014)
Increased prevalence of ovotestes	Wester (1991); Stentiford., *et al*. (2003)
Testicular tumors (Sertoli cell adenoma, fibroplasia and fibrosis)	Granado-Lorencio., *et al*. (1987); Řehulka (2013)
Cellular level	
Germ cell atrophy	Vergílio., *et al*. (2014)
Leydig cell hypertrophy and changes in pycnosis	Kirubagaran and Joy (1992)
Sertoli cell atrophy	Wester and Canton (1992)
Spermatozoa	
Aggregation	Vergílio., *et al*. (2014)
Altered morphology	Vergílio., *et al*. (2014)
Arrested sperm development or reduced abundance	Wester 1991; Wester and Canton (1992); Miles-Richardson., *et al*. (1999); Rajan and Kuzhivelil (2013); Vergílio., *et al*. (2014)
Necrosis (pycnosis and karyorhexus) or apoptosis	Wester and Canton (1992); Miles-Richardson., *et al*. (1999); Blazer (2002); Drevnick., *et al*. (2006)

Table 1: *Histopathological effects associated with mercury in the testes of fishes.*

The tracing of Hg to muscle and organs of fishes has used various methods. Intraperitoneal injection of the radionuclide $^{203}Hg(NO_3)_2$ resulted in accumulation the nuclide in the gall bladder, spleen, eye, kidney, intestines, and gonads (Weisbart, 1973). $^{203}HgCl_2$ and $CH_3{}^{203}HgCl_2$ in seawater was traced to bulk samples of blood plasma, red blood cells, bone, muscles, and various visceral organs including ovaries and testes (Pentreath, 1976a, b). Histochemically, Hg was traced with autometallography (AMG) to the kidney, hepatopancreas, spleen, and intestine of fishes (Kaewamatawong., *et al*. 2013). Zarnescu (2009) used AMG to trace Hg to ovarian tissues and oocytes of a fish, and confirmed localization of Hg with immunochemistry.

Hg was traced through the liver to the ovaries with AMG by Govoni., *et al*. (2017). In the liver, Hg-sulphide granules were observed throughout the cytoplasm of hepatocytes, primarily near the nucleus. In the ovaries, granules were observed in tissue and cells of the ovarian stroma, in developing oocytes, and in spawned eggs of the marine fish Leiostomus xanthurus. Here, we report Hg traced to the testes of the same fish experimentally exposed to dietary Hg as described in Govoni., *et al*. (2017).

Materials and Methods

Experimental design

Methods for the treatment of adult fish were those of Govoni., *et al*. (2017). Groups of adult *L. xanthurus*, were caught in the wild, held in the laboratory for 90d, and fed: (1) a control diet of Finfish Hi-Performance feed pellets; and (2) a treatment diet of ground axial muscle of *Makaira nigricans* with naturally high concentrations of Hg (Barber and Whaling, 1983), mixed with ground pellets. Muscle of *M. nigricans* provided a natural dietary vector for tracing Hg into testes. Hg concentrations in muscle of *M. nigricans* and in ovaries of *L. xanthurus* were estimated in solids and in solution by thermal decomposition, amalgamation, and atomic absorption spectrophotometry using a Milestone DMA-80 Hg analyzer. The concentration of total Hg in muscle of *M. nigricans* was 3.37 µg/g wet weight (WW); the concentration of extracted MeHg was 0.48 µg/g (WW); and by difference, the concentration of HgII was 2.89 µg/g WW. Fish were fed ad libitum for 90 *d. Gametogenesis*, including spermatogenesis was induced by shortening photoperiod and increasing temperature.

Histology and Histochemistry

Sections (5 µm) were cut from treatment and control livers, ovaries, and batches of spawned eggs (Govoni., *et al*. 2017), and from the testes of treatment fish. Sections were treated with AMG following Danscher and Møller-Madsen (1985), with developing solutions and protocols of Danscher., *et al*. (2000), and counter stained with Mayer's Harris's hematoxylin and eosin-y-phloxine. A preliminary study to assess the efficacy of AMG in demonstrating Hg in liver and ovaries with densitometry indicated that Hg-sulfide granules, the reaction products of AMG, were significantly related to total Hg concentration in ovaries (Govoni., *et al*. 2017, Supplement 1). Total Hg in treated ovaries ranged from 0.018 to 1.0 µg/g wet weight. In Govoni., *et al*. (2017), Hg-sulfide granules were demonstrated in hepatic and ovarian tissues and cells of treatment fish, with some granules were evident in fish from control fish, not fed muscle of *M. nigricans*, but the prevalence of granules was greater in treatment fish; the presence of some granules in control fish was attributed to environmental exposure before capture (Govoni., *et al*. 2017).

Nomenclature

Spermatogenesis is complex in fishes and consists of multiple phases and stages with alternate nomenclature (Schultz., *et al.* 2010; Uribe., *et al.* 2014). Here, we apply the simple, standardized nomenclature for phases of Brown-Peterson., *et al.* (2011), with the tissue and cellular definitions of Shaw., *et al.* (2012).

Results

Prevalence of Hg-sulphide granules

All 20 slides prepared from testes (from separate fish) exhibited Hg-sulphide granules, although the extent and distribution in tissues and cells varied among slides, and therefore among specimens. Ten slides exhibited slight distribution with granules scattered in developing germ cells and support tissue around internal lobular sperm ducts, not the main sperm duct. Testicular tissue was in various phases of spermatogenesis, with developing and spawning-capable phases prevalent; one slide was from a testis in immature stage.

Tissue and cellular localization of Hg-sulphide granules

Hg-sulphide granules were evident in spermatogenic germ cells, epithelial cells and connective tissue surrounding sperm ducts, Sertoli cells surrounding spermatocytes, and in spermatozoa. Granules were widespread in spermatogenic cells and in the epithelial cells that surround sperm ducts (Figure 1). In testes that were in developing phase, Sertoli cells in the germinal epithelium of spermatocytes exhibited granules (Figure 2). Sertoli cells associated with either collapsed spermatocytes or collapsed, small, sperm ducts had a high prevalence of granules. Granules were evident in melano-macrophages in interstitial tissue and in association with Sertoli cells (Figure 3). In testes that were in spawning capable phase, spermatozoa within the main sperm duct (Figure 4) and in smaller, radiating sperm ducts within lobular tissue were heavily invested with granules. These spermatozoa appeared necrotic and cellular debris was present within the ducts (Figure 5).

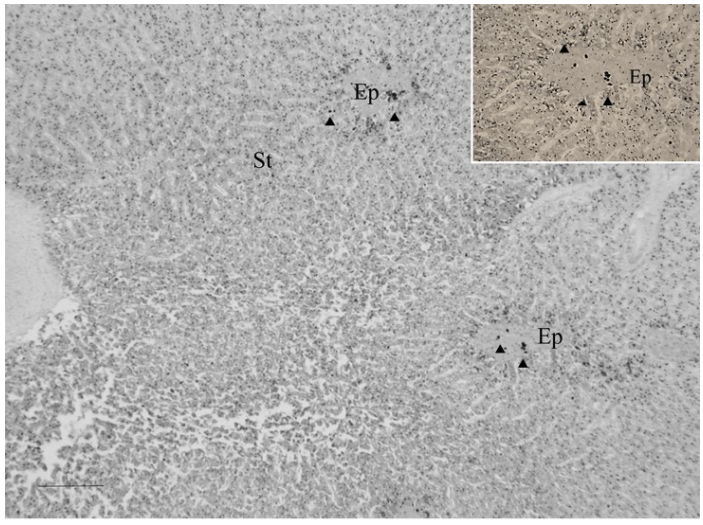

Figure 1: *Widespread distribution of Hg-sulphide granules revealed by auto metallography in spermatogenic tissue and in epithelial tissue in the walls of ducts of the marine fish Leiostomus xanthurus; Hg-sulphide granules in epithelium surrounding sperm ducts (inset). Arrowheads are example Hg-sulphide granules or aggregations of granules; St is spermatogenic tissue and Ep is epithelium; scale bar is 100 µm.*

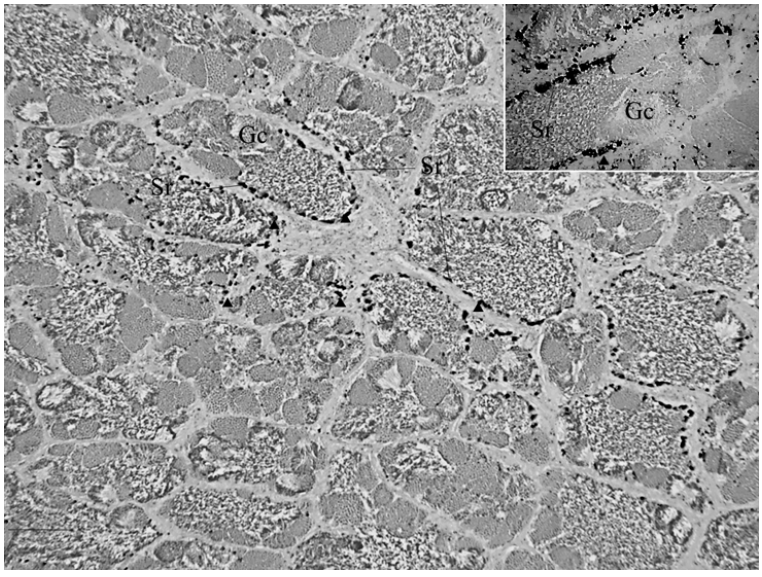

Figure 2: *Hg-sulphide granules revealed by auto metallography in Sertoli cells surrounding spermatogenic tissue with spermatocytes of the marine fish Leiostomus xanthurus; Hg-sulphide granules in a spermatocyst (and inset). Arrowheads are example Hg-sulphide granules or aggregations of granules; Sr are Sertoli cells and Gc are various germ cells; scale bar is 100 µm.*

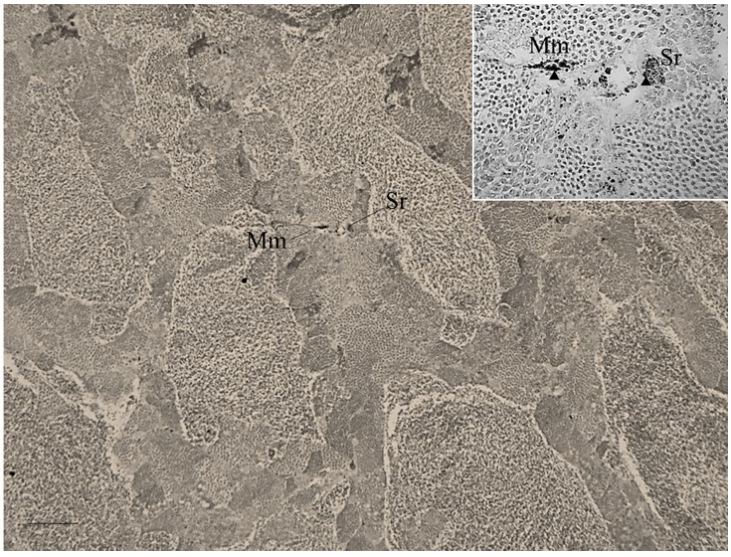

Figure 3: *Hg-sulphide granules revealed by auto metallography in melano-macrophages in interstitial tissue and in association with Sertoli cells of the marine fish Leiostomus xanthurus (and inset). Arrowheads are example Hg-sulphide granules or aggregations of granules; Mm are melano-macrophages; Sr are Sertoli cells; scale bar is 500 µm.*

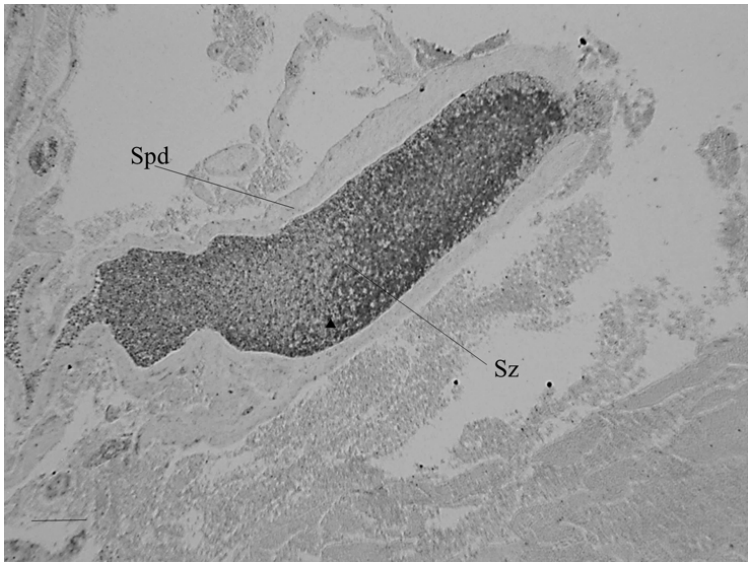

Figure 4: *Hg-sulphide granules revealed by auto metallography in spermatozoa within sperm ducts embedded in the interior of lobules of the marine fish Leiostomus xanthurus and apparent necrotic spermatozoa and cellular debris. Arrowheads are example Hg-sulphide granules or aggregations of granules; Sz are necrotic spermatozoa; scale bar 4500 µm.*

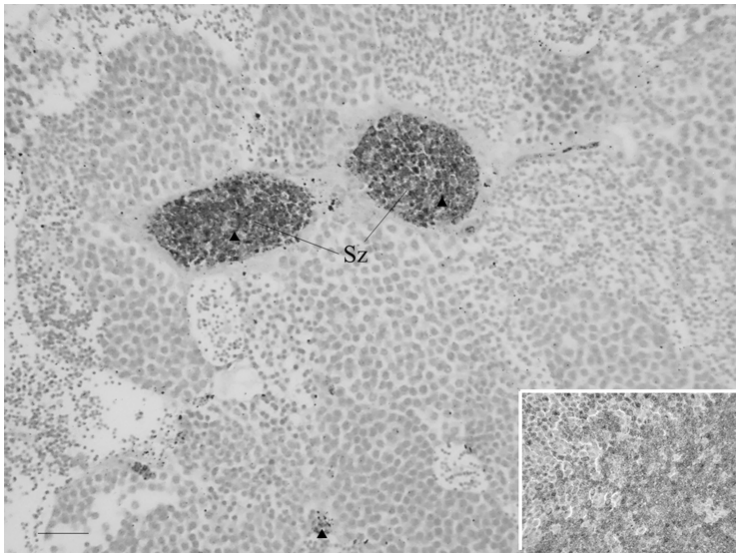

Figure 5: *Hg-sulphide granules revealed by auto metallography in spermatozoa within sperm ducts embedded in the interior of lobules of the marine fish Leiostomus xanthurus and apparent necrotic spermatozoa and cellular debris (inset). Arrowheads are example Hg-sulphide granules or aggregations of granules; Sz are necrotic spermatozoa; scale bar 4500 µm.*

Discussion

Variable feeding by fish on Hg-contaminated food accounts for variation in the extent and distribution of Hg-sulphide granules among slides (and specimens). Observations during experiments indicated variable feeding among fish, although gut content load was not measured when fish were collected for histological preparation.

Auto metallography is a proven methodology that demonstrates Hg in tissues and cells, including testicular tissue, of other vertebrates, along with fishes. With AMG, Danscher and Møller-Madsen (1995) demonstrated Hg-sulphide granules in the testes of rats, where granules are found in Sertoli cells, Leydig cells, and in melano-macrophages in the interstitial connective tissue. Hg has been traced with AMG to melano-macrophages of the liver of frogs (Loumbourdis and Danscher, 2004). Melano-macrophages are common in hepatic (Stentiford., *et al*. 2003), splenic (Kaewamaatawong., *et al*. 2013), and ovarian and testicular tissue of fishes (García-López., *et al*. 2005; Blazer 2002). In livers of fish, proliferation of melano-macrophages is associated with Hg intoxication (Adams and Sonne, 2013). In testes, aggregations of melano-macrophages are associated with the proliferation of Sertoli cells and their phagocytic activity, possibly accelerated by Hg (Blazer, 2002).

The testicular-tissue and cellular deposits of Hg-sulphide granules in *L. xanthurus* coincide with areas where Histopathological effects are evident in the testes of other fishes (Table 1). In *L. xanthurus,* Hg-sulphide granules were evident in the germ cells, epithelium surrounding sperm ducts, in Sertoli cells in the lining of spermatocytes, in spermatozoa, and possibly in melano-macrophages. Testicular tissues, where granules were visible, often lacked cyst Arian structure, a histopatholical effect recognized in other fishes (Table 1). Sertoli cells surround cysts through their cytoplasmic extensions and thereby define spermatocytes structurally, support the germ cells throughout spermatogenesis, and phagocytize residual bodies left from the transition of spermatids to spermatozoa, as well as residual spermatozoa after spawning (Schulz., *et al*. 2010; Uribe., *et al*. 2014). Disruption of the function of Sertoli cells might account for the lack of cyst Arian structure observed in some of the testes examined for L. xanthurus. Apoptosis is observed in the ovaries (Drevnick., *et al*. 2006) and testes (Řehulka, 2013) of fishes. Some apoptosis is normal in fish spermatogenesis (Vilela., *et al*. 2003; Nóbrega., *et al*. 2009),

yet necrosis and apoptosis of is also stressor-induced (Yabu., *et al*. 2001). Hg-induced proliferation of Sertoli cells is also evident (Miles-Richardson., *et al*. 1999). Necrosis and apoptosis might account for the collection of necrotic spermatozoa and cellular debris found in spermatic ducts (Řehulka, 2013).

Fishes take up Hg from the environment directly as HgII, and through their food, primarily as MeHg. Among all tissues and organs of fishes, Hg is redistributed minimally to the organs of reproduction (Johnston., *et al*. 2001; Kasper., *et al*. 2009), and the least to testes (Pelletier and Audet, 1995). Yet, AMG demonstrated Hg-sulphide granules in the testicular tissues and cells of a marine fish, *L. xanthurus*. Concomitant, Histopathological effects that coincide with Hg-induced Histopathological effects in other fishes and that compromise reproduction were observed.

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