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Full Length Research Paper

Stress evaluation in dourado females (*Salminus brasiliensis*) submitted to two different methods of induced spawning

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The goals of this study were to evaluate dourado female egg viability submitted to stress condition caused by two different reproductive induction methods, extruded and semi-natural. Sixteen females randomly chosen were induced with pituitary extract and allocated in two groups; the first, in which oocytes were manually extruded and the second group, females were allowed to naturally spaw. Blood samples were collected for glucose, cortisol and hematocrit determinations as well as for red blood cells and white blood cells smears evaluations. A control group had its blood collected before hormonal induction. Fertilization rate was 40.6 and 91.7% for extruded and semi-natural methods, respectively, and the survival rate was higher in semi-natural groups. Both experimental groups showed higher glucose and cortisol levels compared to control group. The results for hematocrit, hemoglobin concentration and erythrocytes numbers did not differ among groups. Extruded and semi-natural procedures elevated monocytes percentage. In conclusion, the semi-natural procedures are more efficient than extruded method and should be taken into account for *Salminus brasiliensis* handling of breeders.

Key words: Fish breeders, reproductive methodology, hematological parameters.

INTRODUCTION

Dourado, *Salminus brasiliensis* (Cuvier, 1816), from Characidae family, is broadly distributed at Pantanal, Paraná, Uruguay, São Francisco watersheds as well as at Lagoa dos Patos associated-basin (Morais and Schubart, 1955; Gomes et al., 2003). It is a carnivorous fish, found in lotic environmental and performing ascending reproductive migrations (Streit et al., 2007). Zaniboni and Schulz (2003) assigned the dwindling of this specie in nature to riparian deforestation, fishing and capture of young specimens, drainage of adjacent lagoons and alterations of hydrological regime caused by dams' construction, water contamination and nonnative

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species introduction. Hence, *S. brasiliensis* is candidate for artificial reproduction programs for aquaculture preservation purposes.

More recently, some studies have demonstrated environmental factors-induced cortisol releasing to play crucial role in physiological response during the onset of sexual development and increasing steroids levels (Solomon-Lane et al., 2013; Nozu and Nakamura, 2015); however, the exact mechanism involved in cortisol signaling is still unclear (Goikoetxea et al., 2017). During induced reproductive process, the fertilization rate of *S. brasiliensis* is considered limited, which can be associated with inadequate procedures, commonly applied to exotic species reproduction (Zaniboni-Filho and Weingartner, 2007). Eggs quality and viability are directly related with reproductive stress (Zanoni et al., 2016).

Handling of fish breeders during hormonal induction leads to morphological, biochemical and physiological alterations that can be characterized as a stress condition (Eslamloo et al., 2014). In this context, fish stress monitoring through attendance of physiological conditions is an important and valuable tool for aquaculture. The studies of Mazeaud et al. (1977), Barton and Iwama (1991) and Schreck (2010) proposed that fish physiological stress response initiates with stressor agent perception, which leads to hypothalamic-pituitary-interrenal axis catecholamines releasing. The elevated levels of these hormones in turn result in a secondary physiological response that influence other organs and systems causing osmotic status, immunological status and bioenergetic use to change, leading to important alterations of reproductive capacity.

Hematological parameters are important tools to evaluate fish physiological status, may serve as an indicator of stress and reflects the associated pathological alterations (Fazio et al., 2012; Zanoni et al., 2016). To recognize these alterations and to compare them with reference values for a determined species allow to quantify the duration and the severity of stress (Fazio et al., 2015). In addition, hematological profile has been used as biomarker for aquaculture (Tavares-Dias et al., 2007) and able to validate stress conditions in fish. Blood sample analysis predict pathological alterations in an organism since hematopoietic process is under influence of several biological and environmental factors (Tavares-dias et al., 1999). Hematocrit alterations such as red blood cells numbers and hemoglobin levels (Graham et al., 1985), are normally investigated in this context. The RBC elevation results from the releasing of young erythrocytes caused by spleen contraction (Caldwell and Hinshaw, 1994). Yet, there is a considerable variation in the results found by different authors.

Among blood figurative elements, leukocytes play crucial role in nonspecific immunity, and changes in its value can be attributed to the fishes' welfare (Misra et al., 2006). Particularly, leukocyte profile is useful as a

physiological indicator of stress. Plasma glucocorticoids disturbance can increase neutrophil numbers (neutrophilia) and reduce limphocyte concentrations (lymphocytopenia), and as result, the proportional neutrophil to limphocyte ratio is positively associated to the intensity of stressor agent as well as to the levels of circulating glucocorticoids. Some evidences also point to infections and diseases caused by cortisol excess to influence neutrophil to lymphocyte ratio (Davis et al., 2008).

In order to put forward conservational aquaculture, there is an increasing need to achieve higher breeder survival indexes and improve quality of their eggs. During induced reproduction, maintenance of physiological homeostasis and minimization of stress procedures could lead to an increased number of healthy fish fry. The aim of this study was to evaluate the fertilization rate, survival, cortisol and glucose concentrations; and, hematological parameters from dourado breeders underwent two different induced reproduction procedures; semi-natural and extruded methods.

MATERIALS AND METHODS

The experiment was conducted with 32 males and 16 females of dourado randomly chosen from the broodstock of Hydrology and Aquaculture Station of Duke Energy International, located at Salto Grande, Sao Paulo State, Brazil (49° 130 W and 23° 100 S).

From December 2009 to January 2010, specimens were captured by trawling, selected based on their external indicators of gonad maturation (hyperaemic urogenital papilla, bulged and soft womb) and transported to laboratory with 50 L plastic bags, containing 1/5 water. Then, fish were anesthetized with 1 g/100 L of water benzocaine solution, and weighed. The body weight was used to calculate the pituitary extract dosage. After these procedures, breeders were transferred to maintenance tanks and kept under 28°C, with constant water flux to anesthesia recovery. To reproductive induction processes, it was utilized carp pituitary gland extract macerated in porcelain crucible and diluted in saline (0.9%) at room temperature. Females received two intraperitoneal applications, one considered preparatory of 0.5 mg/kg, injected near the pectoral fin peduncle using a 5 ml syringe and hypodermic needles and the second one, 5 mg/kg, 12 h after the former. Male dourado were injected a single dose of 2.5 mg/kg pituitary extract at the time of the second dose of females.

For semi-natural procedures, 8 females and 16 males were randomly transferred to an external, circular tank, with 5.1 m radius and 2.0 m deep, receiving constant flow of 131 L/s of water, at 28°C. This architecture creates an unidirectional water flux, in such a way that the eggs could be collected by a drainage system at the bottom of the tank, communicating directly with a 200 L Woynarovich' incubator, with continuous flow of 7 L/s. Retained eggs were collected every hour for a period of 6 h consecutively, then they were taken to the laboratory where they remained in Israelis-like incubators until hatching (Zanoni et al., 2016). The number of eggs was calculated by multiplying the average of three different samples of one mL by the total volume of eggs.

For extrusion procedure, at 8.5 h after the last dose of pituitary extract, all females were anesthetized with benzocaine and the oocytes extruded manually and collected in plastic containers. Two males were randomly chosen, anesthetized and semen collected by extrusion was added to oocytes and mixed dryly. Hydration of oocytes and activation of male gametes were completed by adding

Table 1. Reproductive efficiency indexes and survival from females of dourado submitted to extruded and semi-natural procedures.

Variable	Treatment	
	Semi natural	Extruded
Total volume of eggs (L)	8	12
Estimation of eggs (ml)	46±4	53±6
Fertilization rate (%)	91.7±4.6	73.5±8.2[*]
Survival of female (%)	100	62.5

Data expressed as mean±SD; p≤0.05. Symbol *: Differ significantly from semi-natural group.

water at 28ºC, followed by the transfer to Israelis-like incubators. The number of eggs was calculated as previously described.

For both procedures, the following reproductive efficiency indexes were calculated:

Fertilization rate (FR) = (number of viable eggs/number of total eggs) × 100

To measure the concentration of cortisol, 1 mL of blood was collected by venipuncture in caudal region using 3 ml syringes embedded in EDTA 10% and 28 x 12 needles. Female blood samples of the two experimental groups were collected soon after spawning and control group blood sample was collected soon after fish were captured. Cortisol concentration was determined by ELISA using commercial kit (EIA, 55050, Human-Kit; Cayman Chemical, Ann Arbor, MI, USA) and glucose levels were determined with digital glucometer ACCU CHECK active®. 1 mL of blood was also collected and added to tubes with EDTA 10% for hematological analysis that were carried out at the Clinic Pathology Laboratory of Universidade Estadual do Norte do Paraná – UENP, Paraná, Brazil. Blood smears, in duplicate, were stained with May–Grunwald–Giemsa dye (Rosenfeld, 1947). The indirect total and differential leucocyte count were performed as proposed by Hrubec and Smith (2000). Our results were evaluated by one-way ANOVA and complemented with Tukey, p≤0.05, while data are expressed as mean±SD.

RESULTS AND DISCUSSION

In the semi-natural procedure, females produced 8 L of hydrated eggs, with 46 eggs in each mL, resulting in 368,000 eggs and in extruded procedure, the result was 636.000 eggs, in a final volume of 12 L with 53 eggs/L as shown in Table 1. In both procedures, 100% of dourado females responded to the treatment with pituitary extract. According to Bromage et al. (1994), eggs releasing at the time of spawning is crucial for egg quality; in such way, the release of eggs before or after this event can lead to low fertilization rates and low quality fish larvae (Springate and Bromage, 1985).

Fertilization rate can be an indicative for eggs quality, and immature eggs display small perivitelinic space caused by incomplete cortical activation which in turn can compromise fertilization process and embryogenic development (Kjørsvik et al., 1990; Zanoni et al., 2016). Our results show fertilization rate to be higher in semi-natural method compared to extruded method as described in Table 1. This result corroborates David et al.

(2002), who obtained fertilizations rate of 25.8% for extruded and 94.5% for semi-natural spawning of *Leporinus macrocephalus*, and also by Zanoni et al. (2016) with *Brycon orbignyanus,* who described fertilization rates of 87.9 and 8.17%, for semi-natural and extruded procedures, respectively.

The percentage of females that survived the reproduction procedures was 100 and 62.5% for semi-natural and extruded, respectively. Notably, 24 h after the extrusion processes, all the females of the last group died. The mortality rate after extruded procedure for *S. brasiliensis* is normally high. Sato et al. (1997) related 83.3% death of extruded breeders, which showed several lesions, body hiperemia, bacteria and fungi secundary infections. All these symptoms can be caused by loss of scales during fish manipulation (Elaine et al., 2002), and as in our case could, in part, explaining our findings pointing the first caution with extruded procedure.

Barton (2002) proposed ideal levels for corticosteroids to be under 5.0 ng/mL of blood, although in fishes, this range between 30-40 ng/mL. In Figure 1, it can be seen that cortisol concentration in control, semi-natural and extrude groups were 11.27±4.26, 22.74±12.93 and 34.9±11.26 ng/mL respectively. These results are in accordance with previous studies from our group and with other authors who have not found differences in this hormonal parameter in fish submitted to stressor conditions (Gomes et al., 2003; Hoshiba, 2009; Zanoni et al., 2016). However, in semi-natural group, the lower cortisol level and higher matrix survival can be attributed to an improved recovering period since the animal were displaced in an external circular tank with bigger space and increased water flow which is similar to what is found in natural environments (Boesoaard et al., 1993; Milligan et al., 2000).

Our outcomes for fertilization and cortisol levels demonstrate the stress effects for eggs quality, which can be explained by cortisol influence on fish reproductive glands. Nepomnaschy et al. (2006) and Whirledge and Cidlowski (2010) showed the necessity of glucocorticoids levels for a normal gonadal function. Elevated levels of cortisol have a negative impact over fertility rates (Carragher and Sumpter, 1990); furthermore, stress itself can suppress reproductive hormones (Pickering et al.,
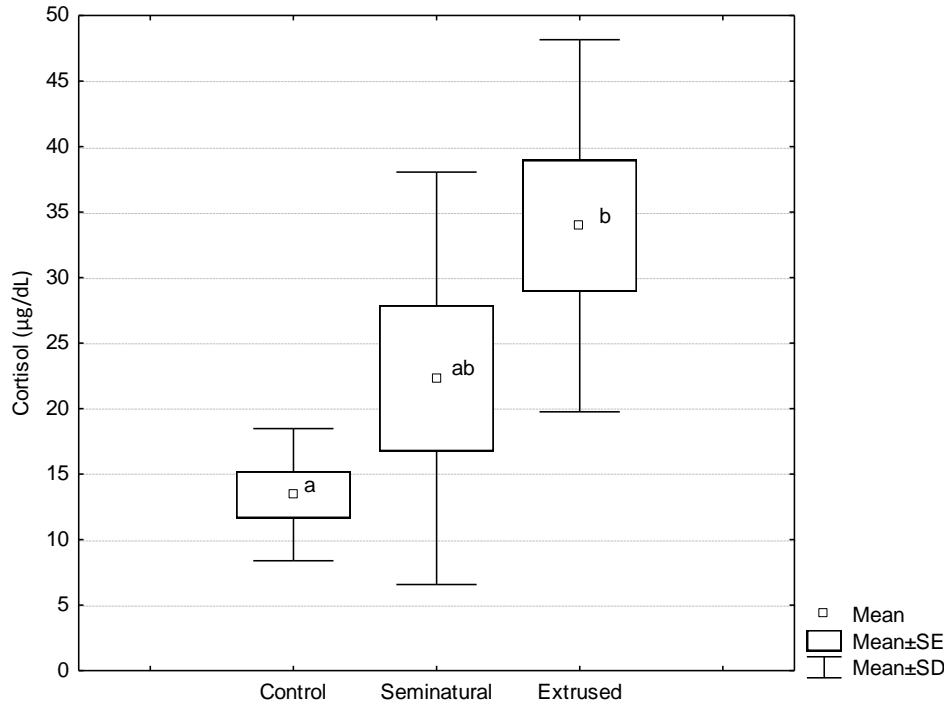
Figure 1. Cortisol level in serum from females of dourado submitted to extruded and semi-natural procedures. Data are expressed as mean±SD, p<0.05. Different letters indicate significant differences between the treatments.
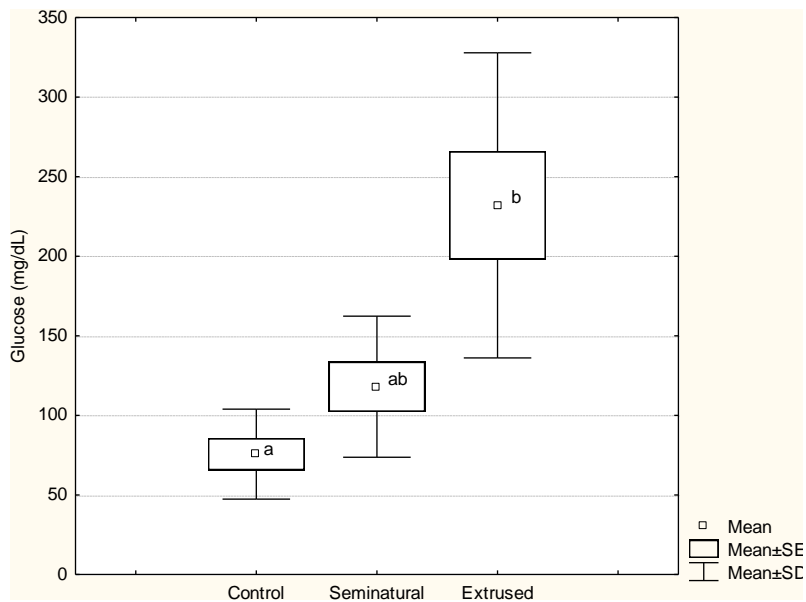
Figure 2. Glucose levels in the serum from females of dourado submitted to extruded and semi-natural procedures. Data are expressed as mean±SD, p<0.05. Different letters indicate significant differences between the treatments.

1987; Pankhurst, 1994; Haddy and Pankhurst, 1999). Extruded procedure increased glucose circulating levels compared to control and semi-natural groups, as it can be seen in Figure 2. Similar response was observed by Gomes et al. (2003) and (Brandão and Levy, 2006), for *Arapaima gigas*, and Gomes et al. (2003) for *Colossoma*

Table 2. Red blood cells parameters from females of dourado submitted to extruded and semi-natural procedures.

Treatment	Variable		
	Haemoglobin (%)	Haematocrit (g/dL)	Erythrocytes (10^6/µL)
Control	49.66 ± 5.8	12.01 ± 0.90	2.9 ± 6.4 x10^5
Extrusion	59.33 ± 10.6	11.36 ± 2.0	2.7 ± 4.86 x10^5
Seminatural	46.5 ± 6.1	13.56 ± 1.3	3.5 ± 3.7 x10^5

Data are expressed as mean±SD, $p<0.05$.

Table 3. Leucocyte series from females of dourado submitted to extruded and semi-natural procedures.

Variable	Treatment		
	Extruded	Semi-natural	Control
No. leucocytes in 1000 erythrocyte	21.3±4.6	20.6±8.3	21.2±4.9
Monocyte	8.4±2.3	13.1±3.12[*]	17.8±4.3*
Lymphocyte	13.0±7.7	9.16±8.4	12.2±2.9
Eosinophil	0.4±0.8	0	0.33±0.81
Neutrophils	11.33±5.71	15.66±9.26	15.4±8.5
Thrombocyte	62.16±18.87	55.3±11.3	63.6±4.15

Data are expressed as mean±SD, $p<0.05$. Different letters indicate significant differences between the treatments. Symbol *: differs significantly from control group.

macropomum. Glucose levels elevation is considered as an adaptive response during the stress response and is related to cortisol and others catecholamine's levels. Taken together, cortisol and glucose levels can be considered good predictors of stress in *S. brasiliensis.*

According to Lucelle et al. (2004), hematological evaluation of fishes during fish farming can reflect its physiological status. Stress conditions increase cortisol concentration modifying physiologic and metabolic status, which can be demonstrated by erithrocytes elevation and mean corpuscular volume (Vosylienė, 1999). Alterations found in erythrogram helps the identification of anemic conditions, and some alteration seen in leucograms is useful for infectious process and homeostasis imbalance diagnostics. Data presented in Table 2 demonstrate that there is no statistically difference for hemoglobin concentration, hematocrit and number of red blood cells comparing experimental groups. Similarly, Abreu and Urbinati (2006), for *Brycon amazonicus,* Martins et al. (2004), for *Oreochromis niloticus* and Pimpão (2006), for *Ancistrus multispinis,* found no difference in hematocrit in fish under stress conditions. Number of red blood cells and hemoglobin concentration, otherwise, differ from Das and Mukherjee (2003) and Adhikari et al. (2004), who demonstrated reduction in these parameters for *Labeo rohita* exposed to stressor agents. It can be assumed that, for dourado, semi-natural and extruded methodologies do not alter eritropoietic process, besides that, for the same species, different results for hemoglobin concentration and hematocrit can be caused by blood samples collection techniques, including anticoagulants and anesthetic use, time elapsed between collection and laboratorial analysis, among others (Tavares-Dias and Sandrim, 1998). More studies are needed to the understanding of these differences.

Teleosts blood composition depends on physiological and ecological factors such as sex, gonadal development stage, stress, infection and environmental conditions. Leukocytes number vary among families, genus and species, and it is related with the surrounding environment (Tavares-dias and Moraes, 2004). There was no difference in total leukocytes when comparing both methods as described in Table 3. According to Abreu and Urbinati (2006), feeding *Brycon cephalus* breeders with different concentrations of vitamin C submitted to air exposure, and Pimpão (2006), studying *Ancistrus multispinis* exposed to deltametrin, found similar results. For the most of fishes' species, leukocytosis can be found as soon as a stressor agent is present in attempt to recover from homeostasis disruption, while leukopenia is generally attributed to diseases that affect immunological system (Vosylienė, 1999). In this study, leukocytes total number was unaltered despite cortisol levels alterations. Nevertheless, monocytes number was higher in both experimental groups compared to control as shown in Table 3. This cell type acts in inflammatory responses involving phagocytosis process (Dalmo and Bøgwald, 2008; Salvador et al., 2013). The result demonstrated

here point the reproductive induction process in Dourado to be considered an inflammatory stimulus, recruiting monocytes for local tissue, since they are considered primary cells for antigens presenting in teleosts.

Neutrophils, lymphocytes and thrombocytes numbers did not differ among groups as seen in Table 3. Stress leads to a lymphocyte redistribution, mainly in lymphoid organs, lowering their circulating levels or, the high levels of cortisol in response to a stressor agent can induce lymphocytes death (Benfey and Biron, 2000). Wojtaszek et al. (2002) confirmed this last alternative by demonstrating accentuated lymphopenia in *Cyprinus carpio* 24 h after cortisol inoculation. Other authors however have related higher number of neutrophils after stress conditions in *Salmo trutta e Salmo gairdneri* (Johansson-Sjobeck and Larsson 1979; Pickering et al., 1987). Thrombocytes number found in our experiment are in accordance with Benfey and Biron (2000). These researches studying *Oncorhynchus mykiss* and *Salvelinus fontinalis* under feedlot and manipulation stress, have verified a slight, but non-statistically significant increase in the number of thrombocytes in fish. This cell type is produced at kidneys and spleen in teleost fishes and play diverse functions, being in a constant flux between circulation and organs (Tavares-dias and Moraes, 2004). Lower number of thrombocytes in circulation could be related to the moment of blood sample collection.

Conclusion

Our result demonstrates *S. brasiliensis* manipulation by utilizing semi-natural procedure or by extrusion causes similar stress characteristics. The improved egg quality obtained by semi-natural procedure is relevant and needed to point out. Taken into account, semi-natural proceeding should be preferred in order to achieve a larger number of healthy larvae.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Microbiota of catfish (*Clarias gariepinus*) tissues harvested from vials polluted with soil from e-wastes dumpsite

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In this study the microbiota of Clarias gariepinus tissues harvested from e-waste-soil polluted vials were assessed. Soil samples contaminated with e-waste were analyzed using standard analytical protocol while the microbial study was obtained using standard conventional microbiological techniques. The results revealed that the soil was sandy-loamy and blackish in colour. High organic matter (17.60%) and organic carbon (10.17%) were obtained. Also, higher calcium (182.00 mg/kg) and phosphorus (146.65 mg/kg) contents compared to other mineral constituents were recorded while, the heavy metals ranged from 0.32- 64.90 mg/kg. Bacterial count ranged from 9.0×10^2 to 4.0×10^3 cfu/L while the fungal count from 4.0×10^2 to 2.3×10^3 sfu/L. The genera of bacteria isolate were identified as Staphylococcus, Proteus, Bacillus, Listeria, Salmonella, Enterobacter, Pseudomonas, Lactobacillus and Corynebacterium and fungal isolates were Penicilliun, Candida, Articulospora, Aspergillus, Rhizopus, Mucor, Zoopage, Varcosporium and Rhodotorula. Microbial species richness of fish tissues from polluted vials indicates a more poly-diverse microbial community compared to those from unpolluted vial. Differences were observed in the occurrence of fungi on the surfaces of catfish tissues in control vial compare to those on fishes from polluted vials. Hence, there is a need for proper water management for fish culturing and caution in the exploration of fishes from polluted natural waters for human consumption.

Key words: Clarias gariepinus, e-waste, fish tissues, microbiota, pollution, microbial species richness.

INTRODUCTION

The United Nations Food and Agricultural Organization predicts that the world's food and feed supply need to grow by 0.7-fold (70%) to sustain the increasing human populace by mid of next century (Bruijn et al., 2018). Currently, the cultivation of aquatic animals is the fastest growing animal food international sector (Bruijn et al., 2018). However, fish production has been hindered by numerous diseases caused by microorganisms and other ectoparasites. Fish tissues house different microbes whose communities is affected by physicochemical water

parameter (such as pH, temperature, nutrient availability, dissolve O_2 and salinity), environmental factors (pollution and season), host age, genotype, feeding approach and rearing conditions (Bruijn et al., 2018). There is growing evidence that microbial consortia rather than single species are linked to fish well-being and diseases (Gilbert et al., 2016). Constituents within man's gut microbiota can substantially promote or suppress disease progress (Gilbert et al., 2016), whereas ecological changes or infections can significantly influence man's gut microbiota by promoting proliferation of opportunistic microbes (Stecher et al., 2013). Similarly, plants' microbial floral plays significant role in the defense against non-living physical and chemical elements (abiotic factors) and the living organisms (biotic factors) stress (Mendes and Raaijmakers, 2015). Likewise, fish microbial flora may possess considerable potential to influences well-being and disease. Owing to the intricate composition of microbial communities, disentangling relationships and identifying species for precise functions is hugely challenging, particularly when environmental impacts on population dynamics and processes are considered (Bruijn et al., 2018). Taxonomical or functional fluxes in the microbiota are fundamental factor for disease propagation or disease defense (Raaijmakers and Mazzola, 2016, Bruijn et al., 2018).

Fish tissues such as the fillet/skin, sensory system (olfactory system), gill system and the gut have contact directly with the surrounding and consequently are the initial points of microbial interaction with their host. More aerobic rather than anaerobic microbes are harbored by the mucus of the fish fillet and gill system (Merrifield and Rodiles, 2015). Microbiota composition of the gills' and skin differs; the protected niches of the gill membrane have more microorganisms that putitatively favor gas exchange (Lowrey et al., 2015). Fish organs such as kidney, brain, liver and muscle under healthy condition are sterile, nonetheless there are scarcity of reports that have studied this in fish in detail (Bruijn et al., 2018). Likewise, there is a dearth of literature on the microbiota and functions of the communities of the fungal fish (Bruijn et al., 2018). Host genetics is also an important influential factor that determine the microbial populations of fish (Ghanbari et al., 2015). The composition of the gut microbiota is similarly depended on the microbiota composition in the environments (water and sediment), in spite of the importance of host genetical make up and feeding approach (Kashinskaya et al., 2015). A recent study shows the gut microbiota of salmon cultured in an aquarium and an open freshwater inlet cage system shared 1:97 operational taxonomic units (OTUs), demonstrating the environment substantially influences the composition of the gut microbiota (Dehler et al., 2017). Another environmental influence shaping the fish microbiota is water component or chemistry, this can be influenced by in flow of substances such as pollutants, into the water environment (Sylvain et al., 2016).

Evidently, these findings show that water condition has vital effects on fish microbiota.

Microbiota is a complex and dynamic population of microorganisms that colonies the tissues of higher organisms, which exert influence on the host during homeostasis and disease (Thursby and Juge, 2017). The collection of microbes, colonising the tissue of higher organism has co-evolved with the host over the years to form an intricate and symbiotic relationship (Thursby and Juge, 2017; Bruijn et al., 2018). Modern techniques for sequencing DNA help to find most of these microbes because many of them cannot be cultured in the laboratory using conventional methods. Microbiota may have a role in auto-immune diseases (Bruijn et al., 2018). Imbalance in the microbial population of the gut may also aggravate digestive or intestinal problems (Willyard, 2011). Since some of the microbes in the body can modify the production of neurotransmitters known to occur in the brain, it may also relieve neuro-chemical imbalances (Bravo et al., 2011; Madigan, 2012). The microorganisms (non-pathogenic) in question, generally they do not cause illness except they grow abnormally. They exist symbiotically with their hosts such as fish (Salvucci, 2014). Disease causing microbes (pathogenic microorganisms) are basic component of fish microbiota, nonetheless their presence does not often lead to illness. On the other hand, when fish mutualistic or commensal microbial community balance is altered, pathogens proliferate and cause infection and disease, this process is known as dysbiosis (Moya and Ferrer, 2016). The imbalances that occur in the defensive symbiotic microbial community could result from changes in the surrounding (environment), including climate and seasonal changes, H_2O conditions, temperature, changes in culturing parameters. Similarly, infection by a primary pathogen can lead to distortion in the microbial balance, this subsequently permits opportunistic disease-causing microbes (pathogens) to invade the host system (Llewellyn et al., 2017). In addition, symbiotic microbes play vital roles that contribute to host health and defense against disease-causing microbes (Xu et al., 2016). Some of these roles among others are direct defensive effects against disease-causing microbes by antibiosis and struggle for limited resources (niche exclusion or omission). Furthermore, these functions can be performed indirectly by inducing the host immune response then nutrient uptake thus promoting fish well-being. Symbiotic microorganisms could stop pathogen infection via several mechanisms, such as niche omission, which involves colonizing mucosal tissues then occupied infection sites and struggling for essential resources (Banerjee and Ray, 2017). The fish microbiota can induce a conserved host response in invasion (colonization) and development, and the fish microbiota communities evidently influences the inflammatory response (Bruijn et al., 2018).

Clarias gariepinus is freshwater fish species of African

origin, is one of the most cultured species in the world (Emiroğlu et al., 2018). This fish species is valuable for fish farming (aquaculture) due to its easy to farm especially in warm climates, fast growth rate, resistance to diseases, and high stocking density (Emiroğlu et al., 2018). Additionally, many catfish species are important human food source. Monitoring of fishes' microbiota such as that of *C. gariepinus* can be useful to evaluate their health status and fitness for human consumption.

Many of the water body in various parts of Nigeria receive huge quantities of untreated effluents and solid waste which contain substances that are harmful not only, to human but also to aquatic biota (Ekpo, 2012). In recognition of deleterious effects of the destruction and loss of habitat caused by solid waste, chemical pollution, eutrophication and climatic alterations on the aquatic organisms, as a result of human activities, combined with an urgent need of a more environmentally sensitive and ecologically sustainable management of water bodies in Nigeria, gingered the assessment of catfish microbiota harvested from polluted vials. Therefore, the main aim of this study is to create awareness on the multi-species diversity and probable health implication of the microorganisms found on the tissue of fish from polluted aquatic environment.

MATERIALS AND METHODS

Collection of samples

Electronic waste (e-waste) contaminated soil samples were collected from Alaba International Market, Lagos, Lagos State (Latitude: 6°27′14″ N, Longitude: 3°23′40″ E, Elevation above sea level: 11 m (36 ft)), Nigeria. E-waste is appliance consuming electricity and reaching the end of its life cycle. E-waste includes a wide range of electronic appliances ranging from large household appliances, such as refrigerators, air conditioners, cell phones, stereo systems and consumable electronic items to computers discarded by their users. E-waste generally contains valued metals such as copper, platinum group as well as potential environmental contaminants such as lead, mercury, nickel, selenium, cadmium, polybrominated diphenyl ethers (PBDEs) and polychlorinated biphenyls (PCBs). Most e-waste is disposed in landfills (Sanusi, 2015). The e-waste burning site is close to the Lagos water body; washing of surface materials from land surface to the sea or leaching of chemicals substances into it can be experienced.

Set up and pollution of vials

Four vials each containing six juvenile catfish were set up and polluted with three different concentrations of soil sample from e-waste dumpsite (0.025, 0.050 and 0.075 kg). This was carried out in the ratio of 1:1, 1:2, 1:3 of water to soil sample (25 L: 0.025 kg, 25 L: 0.050 kg, 25 L: 0.075 kg) after acclimatization of the fishes for 42 days. The fourth vial was not polluted it serves as the control. At the end of five weeks (35 days) bacteria and fungi were then isolated from both the vials and harvested fish tissues (skin, gill, intestine, kidney and liver).

Physiochemical parameters of soil sample

The pH of the soil samples and water samples in the aquaria were determined by the method described by Hendershot et al. (1993). From the soil samples, 10 g were weighted into a 100 ml beaker and 20 ml of distilled water was added to it. The mixture was stirred properly and allowed to stand for 30 min. 20 ml of the water samples were measured into a beaker and the electrode of the calibrated pH meter was dipped into sample water, the observed pH was read and recorded.

Extraction of heavy metals from soil samples was by acid digestion (Yusuf et al., 2015). The digestion was carried out with 20 ml of mixture of concentrate $HClO_4$ and HNO_3 at ratio 2:1 (v/v) on a hot plate and the mixture heated to almost dryness. 20 ml of 0.5 M HNO_3 were added and the solution filtered into 50 ml volumetric flask through Whatman No.42 filter paper. The filtrate obtained was made up 50 ml mark with distilled water and used for heavy metal determination against those of the blank and calibration standards using a flame atomic absorption spectrophotometer (AAS), Apha 4 model. Phosphate concentrations in soil sample was determined using the phosphovanadomolydate colorimetric method (AOAC, 2019). Nitrogen determination was determined by direct nesslerisation method (AOAC, 2019). Organic carbon and organic matter determination were obtained using the methods describes by AOAC (2019).

Total plate count

Samples were taken from fish tissues using sterile swab sticks which were then placed in appropriate diluent for further studies. Plates in triplicates from harvested catfish tissues were observed for their bacteria and fungi loads. Colony and spore counting were carried out by counting the number of visible colonies and spores that appears on the plates for bacteria and fungi respectively. Calculation of colony forming unit (cfu) per ml for bacteria and spore forming unit (sfu) per ml for fungi were based on the dilution factor used.

Tentative microbial identification

Biochemical and morphological identification of bacteria isolates

Individual colonies were identified morphologically and by biochemical test using techniques described by Jesumirhewe et al. (2016). The biochemical tests performed to tentatively identified the bacterial isolates were; gram staining, catalase test, spore staining, motility test, starch hydrolysis, coagulase test and sugar fermentation test.

Identification of fungi

Probable fungi isolates were identified based on the cultural, morphological and microscopic examination of the colonies grown on potato dextrose agar (Onions et al., 1981). The parameters such as colony color, features of hyphae and shape of mature fruiting bodies were microscopically determined. The microscopic observations made were used in identifying the fungi isolate.

RESULTS AND DISCUSSION

The bacterial counts from the harvested tissues were observed to be highest on the skin (3.7×10^3) and the gills (4.0×10^3) (Figure 1). This is due to the constant interaction of these two tissues with the water environment. Although difficult to estimate and compare,
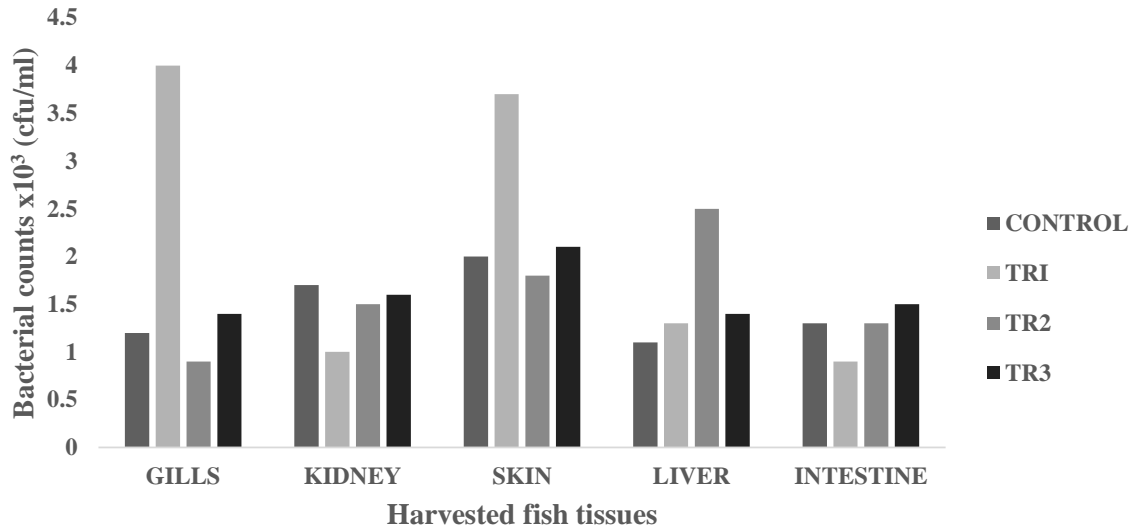
Figure 1. Bacterial count (cfu/ml) of harvested catfish tissues after 35 days.
Legend: TR1 – Polluted with 0.025 kg of e-waste soil, TR2 – Polluted with 0.050 kg of e-waste soil, TR3 - Polluted with 0.075 kg of e-waste soil.

Figure 2. Fungal count (sfu/l) of harvested catfish tissues after 35 days.
Legend: TR1 – Polluted with 0.025 kg of e-waste soil, TR2 – Polluted with 0.050 kg of e-waste soil, TR3 - Polluted with 0.075 kg of e-waste soil.
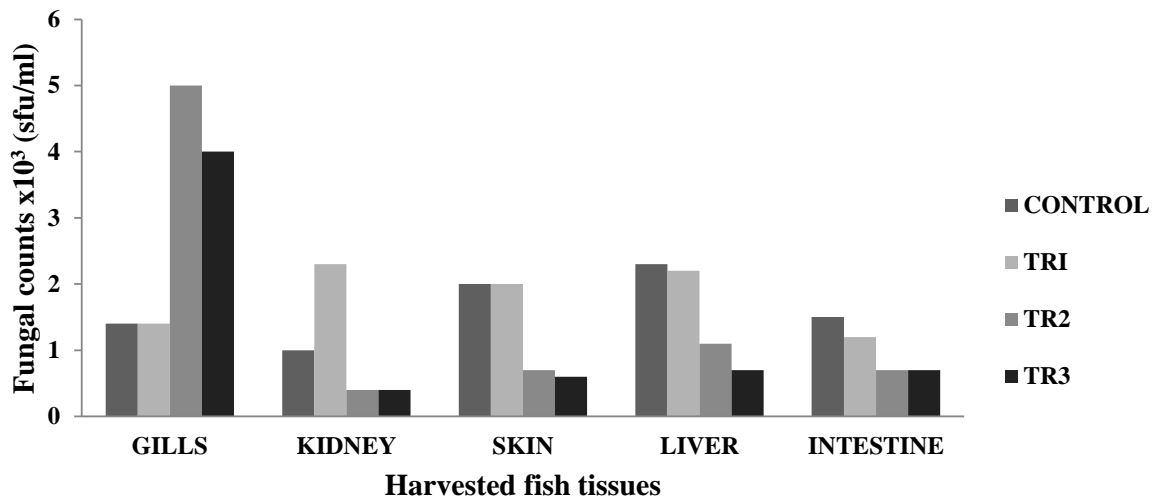
studies have shown bacterial population in similar ranges (Austin, 2006; Merrifield and Rodiles, 2015). Bruijn et al. (2018), reported fish skin typically harbors 10^2-10^4 bacteria per cm^2, whereas the gills harbour 10^3-10^6 bacteria per gram of tissue based on fish species, environment and cultivation methods. The lowest bacterial counts of 9.0×10^2 was observed in the intestine. The low bacterial population in the intestine can be attributed to the anaerobic condition around the gastrointestinal tract that will only support the growth or proliferation of anaerobic or facultative bacteria only (Ashlee et al., 2008). The fungal count followed a pattern which showed fungal population reduces with increase in the concentration of pollutant in the vials (Figure 2). This suggests that fungal proliferations were largely influenced by the presence of the pollutant. Sylvain et al. (2016), reported water chemistry, an environmental factor that can be influenced by pollution, also determines the fish microbiome composition. Microbial communities in natural aquatic environments respond rapidly to changes in their immediate environment (Bentzon-Tilia et al., 2016). For instance, the gut microbiota of salmon cultured in an aquarium facility and in an open freshwater inlet birdcage system, indicates the environment

Table 1. Soil physiochemical parameters.

Parameter	Values
pH	7.90
Organic matter (%)	17.60
Organic carbon (%)	10.17
Organic nitrogen (%)	0.35
Organic phosphorus (mg/kg)	146.65
Lead (mg/kg)	64.90
Cadmium (mg/kg)	0.32
Zinc (mg/kg)	35.50
Cobalt (mg/kg)	0.83
Chromium (mg/kg)	0.54
Manganese (mg/kg)	18.60
Nickel (mg/kg)	2.82

substantially influences the composition of the gut microbiome (Dehler et al., 2017).

Electronic waste (e-waste) soil polluted vials had higher number of microbial isolates comparison to the unpolluted vials. This trend was the same for microbes from fish tissues harvested from the polluted vials; where fish tissues from polluted vials showed more species richness (diversity of microbial species) than those from unpolluted vials. These could be attributed to the organic contents of the e-waste soil sample (Table 1), which promoted and supported the growth of those microbes in the polluted vials (Bentzon-Tilia et al., 2016). This increased species poly-diversity of microbial community of the polluted vials and fish tissues from polluted vials might impact microbial balance in the polluted vials, on the fish and its tissues. The microbial imbalance can lead to unhealthy relationships within the microbes' community (such as parasitism and inhibition effect) and between the fish and the microbes. The relationship between the fish and these microbes could also be pathogenic. Fungus such as *Rhodotorula* has been reported to cause infections in aquatic organisms. Fermanda and Luciano (2012), documented that *Rhodotorula* causes skin infections in both terrestrial and aquatic animals such as chickens, sea animals and lung infections and otitis in sheep and cattle. Dantigny et al. (2005), reported that some of these fungi secrete aflatoxins and other substances that can inhibit the growth of other microorganisms which could lead to declination of bacteria or fungi population. Also, the microbial colonization in and around the gastrointestinal tract of the fish could influence the microbial composition of the digestive tract. This might be of positive effect such as probiotic benefits. Symbiotic gut microorganisms similarly aid the fish in nutrient acquisition (Borrelli et al., 2016). The gut microbes can release exogenous enzymes to promote the digestion of food and degradation of chitin, protein, starch and other large and complex molecules (Montalban-Arques et al., 2015).

Similarly, they produce vitamins and eicosapentaenoic acid that is vital for metabolism to improve the well-being of the host. On the other hand, the effect might be negative, which can lead to poor feeding, impact digestibility rate and fish weight loss. Imbalance in the mix of microbial populations of the gut can also promote or aggravate intestinal problems (Ashlee et al., 2008; Willyard, 2011).

Bacteria isolates from the fish environment (water) were similar to those isolated from the fish tissues (Tables 2 to 3). The microorganisms associated with fish or their tissues are usually dictated by the environment the fish lives (Ekpo, 2012; Dehler et al., 2017). Many researchers (Sugita et al., 1997; Shewan, 2000; Okaeme, 2006, Bruijn et al., 2018) have isolated different species of bacteria from the skin of fresh water fish including *Bacillus* species from the skin of sea water fish. Sugita et al. (1997), reported that *Staphylococcus* spp, *Escherichia coli* were isolated frequently from the skin of fresh water fish while some researchers concluded that predominant genera are *Pseudomonas*, *Staphylococcus* and the member of the family *Enterobacteriaceae* on the skin of fresh water fishes, these agrees with their implication in this study.

Eleven individual isolates of bacteria and twelve fungi (Tables 2 to 7) were isolated from polluted fish aquaria and harvested fish tissues. *Staphylococcus aureus, Bacillus cereus, Bacillus subtilis, Bacillus megaterium, Listeria monocytogenes, Lactobacillus bulgaricus, Pseudomonas aeruginosa* and *Corynebacterium fascians* have been implicated as normal fish floral which are dependent on the environment in which the fish lives, cultured or fish feed (Gram et al., 2000). *S. aureus, Salmonella* sp, *P. aeruginosa, Aspergillus flavus, Mucor mucedo* are linked to fish spoilage (Ashie et al., 1996, Gram et al., 2000, Doyle, 2007; Krijgsheld et al., 2013). Their presence can be attributed to the mortality of some of the catfish at the early stage of the study. Furthermore, *Bacillus* spp*, P. aeruginosa, Articulospora inflata, Zoopage nitospora, Varicosporium elodeae, Penicillium italicum, Aspergillus niger, Aspergillus flavus, Rhizopus stolonifer, Mucor mucedo, Candida* sp and *Rhodotorula* sp have been implicated in other environments such as petroleum polluted environment, gastrointestinal tract, agrarian soil, thus their isolation in the current study demonstrates or suggests their ability to survival under different environmental conditions and substrate utilization versatility (Ashlee et al., 2008; Alfreda and Ekene, 2012; Krijgsheld et al., 2013; Fermanda and Luciano, 2012).

Conclusion

This study demonstrates the diversity of catfish microbial populations elucidated with laboratory scale polluted aquarium system and discusses the potential implications of these microbes. Microbial species richness of fish

Table 2. Probable bacterial isolates from control vial and vials polluted with e-waste soil.

Isolates	Control vial	Polluted vials
Staphylococcus sp	+	+
Proteus vulgaris	+	+
Bacillus cereus	+	+
Bacillus subtilis	+	+
Bacillus megaterium	-	+
Listeria monocytogenes	+	+
Salmonella sp	+	+
Enterobacter sp	-	+
Pseudomonas aeruginosa	-	+
Lactobacillus bulgaricus	-	+
Corynebacterium fascians	-	+

Legend: + = present and - = Absent.

Table 3. Isolated fungi from the control vial and e-waste soil polluted vials.

Isolate	Control vial	Polluted vials
Penicillium italicum	+	+
Candida sp	+	+
Articulospora inflata	+	+
Aspergillus niger	-	+
Mucor mucedo	-	+
Zoopage nitospora	-	+
Varicosporium elodeae	-	+
Rhodotorula sp	-	+
Aspergillus paraciticus	-	+

Legend: + = present and - = Absent.

Table 4. Probable bacterial isolates from tissues of harvested catfish from control vial.

Isolate	Gills	Kidney	Skin	Liver	Intestine
Bacillus cereus	+	+	+	+	+
Salmonella sp	-	-	+	-	-
Bacillus subtilis	-	-	-	+	+
Staphylococcus sp	+	-	+	-	-
Listeria monocytogenes	+	+	+	-	+
Pseudomonas aeruginosa	+	+	+	+	-
Lactobacillus bulgaricus	-	+	+	-	-
Corynebacterium fascians	+	-	+	-	-

Legend: + = Present, - = Absent.

tissues from polluted vials indicate that pollution increased the microbial colonization of these fish tissues. There are also differences in the occurrence of the microbes on the tissues of fish from the control vial and the polluted vials. These might not be of wholesome benefit to the fish.

Overall, fish microbiota has enormous potential for fish health and disease, helping the host in its defense against pathogen colonization and infection or promote disease development leading to its immune defense being overwhelmed.

Table 5. Probable bacterial isolates from tissues of harvested catfish from polluted vials.

Isolate	Gills	Kidney	Skin	Liver	Intestine
Bacillus cereus	+	+	+	+	+
Salmonella sp	-	-	+	-	-
Bacillus subtilis	-	-	-	+	+
Staphylococcus sp	+	-	+	-	-
Listeria monocytogenes	+	+	+	-	+
Pseudomonas aeruginosa	+	+	+	+	-
Lactobacillus bulgaricus	-	+	+	-	-
Corynebacterium fascians	+	-	+	-	-

Legend: + = Present, - = Absent.

Table 6. Fungi isolated from tissues of harvested catfish from control vial.

Isolate	Gills	Kidney	Skin	Liver	Intestine
Penicillium italicum	+	+	+	+	+
Candida sp	+	+	+	+	-
Articulospora inflata	+	-	+	-	+
Aspergillus niger	+	+	+	+	+
Rhizopus stolonifer	+	+	+	+	+
Aspergillus flavus	+	+	-	+	+
Mucor mucedo	+	-	-	-	-
Zoopage nitospora	-	-	-	-	-
Varicosporium elodeae	-	-	-	-	-
Rhodotorela sp	-	-	-	-	-
Trichoderma viridae	+	+	+	-	-

Legend: + = Present, - = Absent.

Table 7. Fungi isolated from tissues of harvested catfish from vials polluted with e-waste soil.

Isolate	Gills	Kidney	Skin	Liver	Intestine
Penicillium italicum	+	+	+	+	+
Candida sp	+	+	+	+	-
Articulospora inflata	+	-	+	-	+
Aspergillus niger	+	+	+	+	+
Rhizopus stolonifer	+	+	+	+	+
Aspergillus flavus	+	+	-	+	+
Mucor mucedo	+	-	-	-	-
Zoopage nitospora	-	-	-	-	-
Varicosporium elodeae	-	-	-	-	-
Rhodotorela sp	-	-	-	-	-
Trichoderma viridae	+	+	+	-	-

Legend: + = Present, - = Absent.

Therefore, proper management of waste disposal, in this case e-waste is necessary. E-waste contamination in aquatic environment can affect the quality of aquatic environment as well as promote microbial proliferation, which could be unhealthy to fishes and also reduce their quality for human consumption.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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