



# Morphological and pathological appraisal of *Huffmanella* sp. (Nematoda: Trichosomoididae) infecting orange-spotted grouper (*Epinephelus coioides*, Hamilton, 1822) at Jubail Province, Saudi Arabia: a case report

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**Abstract** This paper is conducted for identifying the parasite which collected during regular routine inspection for the hygienic condition and health status of fish in 2019 at the central fish market shops at Jubail city, Saudi Arabia. Samples from an orange spotted grouper (*Epinephelus coioides*) fish showed heavy black streaks between the muscle fibers that was considered unmarketable and rejected by the consumer after had been cut and prepared for sealing. The black discolorations were in the form of heavy diffused focal black spots or forming threads of variable sizes embedded in the entire whole-body musculature of the fish. These samples were subjected to parasitological and pathological evaluation. The parasitological examination of the affected muscular tissues revealed infection by *Huffmanella* spp. eggs. The parasitic infection was identified on the bases of the morphological and ultrastructural characters of the eggs. The examination of the affected fish showed multifocal black parasitic remnants between the muscle fibers. The parasitic fragments were distributed all over the fish flesh either superficially in subcutaneous area or deep inside the muscular tissue and they have been concentrated in the flesh around the

vertebral column. In conclusion; this case report confirmed the occurrence of *Huffmanella* spp. infection in the muscles of *Epinephelus coioides* by using light and scanning electron microscopy. Further molecular assessment is recommended.

**Keywords** *Epinephelus coioides* · Granulomatous myositis · *Huffmanella* spp

## Introduction

*Epinephelus coioides* (*E. coioides*; Hamour) was considered as the most preferred fish species consumed in the fish markets by 60–72% of Saudis (Burger et al., 2007). *Huffmanella* spp. (Nematoda: Trichosomoididae, Huffmanellinae) is one of the histozoic nematode parasite infecting marine fishes and less frequently freshwater fishes (Moravec et al., 1998). Furthermore, they are categorized mainly based only on the morphological feature of their eggs, and life-cycle characters such as host family and targeted tissue (Justine and Iwaki, 2014).

Previous studies have shown that *Huffmanella* infection is one of the most common parasitic infections that adversely affect the health of various fish families, worldwide (Eissa et al., 2020). The site of the lesion preference was species dependent, as previously reported for *H. lusitana* infecting the muscles of pouting *Trisopterus luscus* (Ramos et al., 2019), while *H. paronai* was observed in the skin of *Xiphias gladius* (Justine, 2004). Also, two species of *Huffmanellae* were recorded in Japan (*H. japonica* and *H. shikokuensis*) and the eggs of these parasites were found in the flesh of *Upeneus bensasi* and *Stephanolepis cirrhifer* (Moravec et al., 1998).

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The aim of the current study was to identify the causative agent of black muscular tissue discoloration and describing the associated pathological lesions in this fish species.

## Materials and methods

### Study area

The fishes; orange spotted grouper (*E. coioides*); were caught and examined from water of the Arabian Gulf at the eastern region of the Arabian Gulf; at fish landing stations, market shops and fish auctions at Jubail city ('27° 57.9' N and '49° 40' 43.4' E), Saudi Arabia.

### Fish sample

From January 2017 to December 2019, during the routine examination of 850 (280 female and 570 male) orange spotted grouper (*E. coioides*); the examined fish were ranged from 45 to 50 cm with average body weight from 2500 to 2750 kg only one male fish of orange spotted grouper (*E. coioides*) showed intermuscular black discoloration spots like threads. The fresh muscle specimens as well as its internal organs were examined directly under the microscope and different samples were fixed and taken for further parasitological, histopathological and ultrastructural studies.

### Parasitological examination

Different parts of fixed muscle in 10% neutral buffered formalin had multiple black strikes (the black coloration of grade 2) were admitted to the Parasitology Department; Faculty of Veterinary Medicine; Cairo University. These lesions were examined under dissecting Stereoscope and light microscope; further, the scraped eggs from muscles were cleared in lactophenol for one hour, and then mounted in glycerol jelly (Abdelsalam et al., 2020). The measurements of eggs (n = 20) were photographed and measured using an Olympus AX70 connected to a Nikon camera. Measurements of the examined eggs (per microscopic field) were recorded in micrometers ( $\mu\text{m}$ ) with mean (minimum – maximum  $\pm$  standard deviation).

### Ultrastructure studies of eggs using Scanning electron microscope (SEM)

The recovered eggs were prepared by fixation in 2.5% glutaraldehyde at 4 °C for 24 h. Further, the isolated eggs were dehydrated through passing in a grade series of ethanol; each 20 min, dehydrated on filter paper Whitman

no.1. Then, whole dryness was occurred by CO<sub>2</sub> critical point drier (Autosamdri-815, Germany). Finally, the samples were stuck on the stubs, covered with 20 nm gold in a sputter coater (Spi-Module sputter Coater, UK). All the specimens examined and photographed by JSM 5200, Electron probe Microanalyzer, Jeol, Japan at the Faculty of Agriculture, Cairo University, Egypt; number of examined eggs were 10 which photographed by electron microscope (Ruiz et al., 2013).

### Determination of eggs morphology and measurements

The morphological identification of the collected eggs was carried out according to the described keys and available literature (Justine and Iwaki, 2014). The measurements of the different stages of eggs were taken as the length; width of eggs; the length of polar plug; the thickness of the two covering layers of eggs and the thickness of its convoluted larva.

### Histopathological examination

Tissue specimens from infected muscles were fixed in 10% neutral buffered formalin, washed in tap water, dehydrated in ascending concentrations of ethanol, cleared in xylene and embedded in paraffin then sectioned at 5  $\mu$  thicknesses. Hematoxylin and eosin (H&E) stain was performed. All tissue processing procedures were performed according to Heil (2009).

## Results

### Parasitological results

#### Taxonomy

Type host: Orange spotted grouper (*E. coioides*, Ham.); Saudis name “Hamour”.

Site of infection: Intermuscular black spots and/or worm-like threads as a result of presence of the eggs in the musculature.

Type locality: Jubail city on the Persian Gulf coast; Saudi Arabia.

#### Light and scanning electron microscopy of *Huffmanella* spp eggs

All the examined eggs with different stages revealed infection of orange spotted grouper muscles with *Huffmanella* spp. By inspection of muscle 2 stages of eggs were recorded (stage 1: light brown advanced eggs with larva;

stage 2: dark brown advanced eggs with developed larva). All the examined eggs were found to be in advanced stage which had larvae.

Stage 1 (fertilized eggs; n = 10): advanced eggs light brown in color; the eggs was oval to spindle shaped eggs 67.46 (60.3 – 75.6 ± 5.2 µm) in length and the eggs less broad with its width ranged from 30.2 (26.4–36.5 µm ± 3.5). The protruding polar plug ranged from 8.5 (6.5–10.3 ± 1.3 µm); the eggs surrounded with smooth egg envelop and had two layers. The outer brown and inner clear translucent layer with eggshell thickness ranged from 4.3 (3.4–5 ± 0.7 µm). The eggs shell had protuberance. The eggs contained convoluted larva from 3 to 5 coiled within the eggs the larval thickness was 5.4 (4–6.6 ± 0.8 µm); Figs. 1a–e and 2a–d.

Stage 2 (fertilized eggs contain larva; n = 10): more advanced eggs amber brown to dark brown in color; the eggs resembles the eggs in stage 1 but differ in measurements as; the eggs were oval to spindle shaped with 68.3 (62.5–75.3 µm ± 4.9) in length and less broad with its width ranged from 33.2 (30–36.5 µm ± 2.4). The polar plug ranged from 6.8 (6.3–9.2 µm ± 1); the eggs surround with smooth egg envelop; Figs. 1b and 2a and had two layers outer brown and inner clear and translucent layer with eggshell thickness ranged from 4.2 (3–5.4 µm ± 0.6); Fig. 2b. The eggshell had protuberance which as stage 1; Fig. 2b–c. The eggs contain convoluted larva from 3 to 4 coiled, the larval thickness 5.6 (4.5–6 ± 0.4) in diameter; Fig. 1c–e.

## Pathological results

### Gross lesions

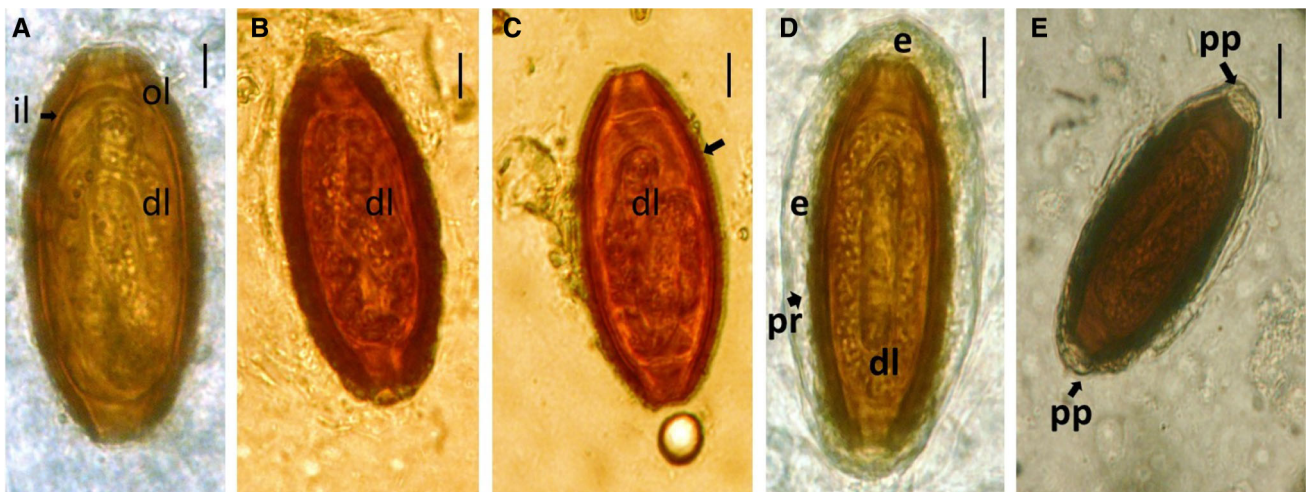
The examination of the affected fish showed multifocal black parasitic remnants between the muscle fibers. The parasitic fragments were distributed all over the fish flesh either superficially in subcutaneous area or deep inside the muscular tissue and they have been concentrated in the flesh around the vertebral column. The examined fish and affected muscles were presented in; Fig. 3a and b.

### Histopathological findings

The examined fresh tissue specimens using the light microscope showed multiple rounded to oval black eggs together with larvae deeply embedded between the muscle fibers. In such areas, granulomatous reaction accompanied with mononuclear inflammatory cells infiltration and fibrous connective tissue proliferation was observed; Fig. 3c.

## Discussion

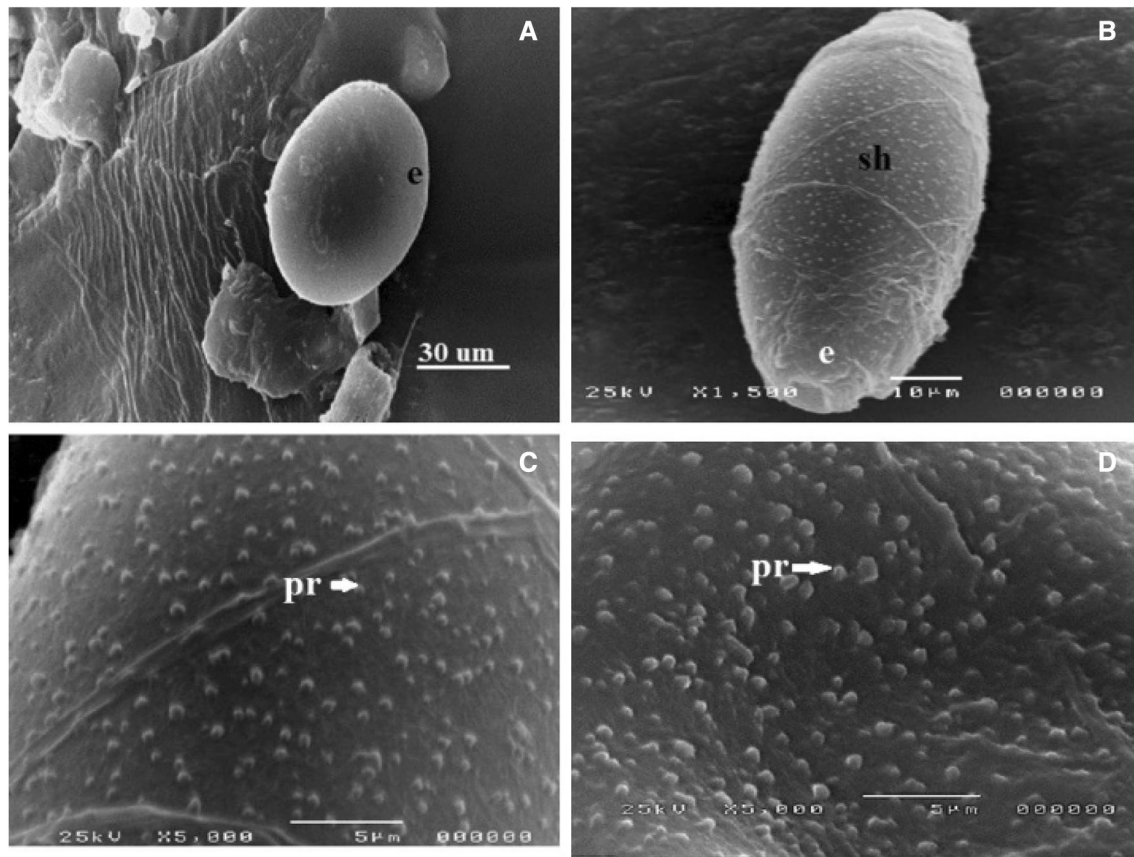
Based on the previous published data; there are about 21 species of *Huffmanella* spp. were recorded worldwide till now; these species were identified on the basis of various morphological criteria as the eggs morphology (egg length and width; shape of shell smooth or spiny either, contain



**Fig. 1** Developmental stages of *Huffmanella* spp. eggs from Orange spotted grouper; **a** stage 1: amber-shelled egg with bilayer eggshell observable with outer layer (ol) dark colored and innermost typically (il) translucent with developing larva (dl), scale bar: 10 µm. **b, c** stage 2: fully developed brown-shelled egg with developing larva (dl); note protuberance in outer layer (arrow); scale bar: 10 µm. **d** stage 2: fully

developed brown-shelled egg with developing larva (dl); note protuberance (pr) in outer layer, the eggs surrounded by smooth envelop (e); scale bar: 15 µm. **e** stage 2: fully developed brown-shelled egg with developing larva (dl); note protruding polar plug (pp); scale bar: 20 µm





**Fig. 2** Developmental stages of *Huffmanella* spp. eggs from Orange spotted grouper; scanning electron micrographs. **a**: Eggs of *Huffmanella* spp. note smooth envelop (e). **b** Eggs showing its shell (sh) had protuberance (pr) and remnant of envelop (e). **c** Higher

magnification of eggs showing protuberance (pr). **d** Higher magnification of *Huffmanella* spp. eggs showing the eggshell surface with protuberance (pr) on it which appear as irregular protrusion

ridges or filaments; with envelop or not; the envelop character, which is spinose or aspinose; Moravec and Fajer-Avila, 2000). Additionally, the identification was done according to habitat of eggs either skin; gills; muscles; swim bladder; within bones; serosa of intestine and liver. Also, identification was carried out on the basis of specific host species.

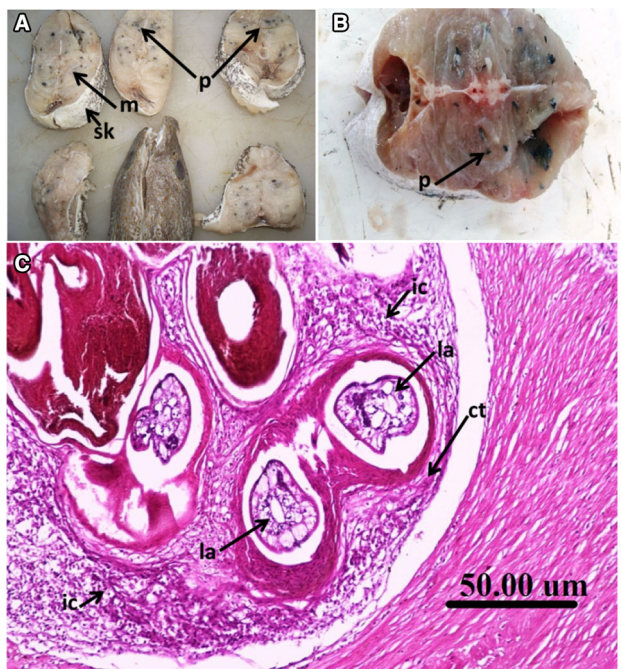
This study was applied on Orange spotted grouper (*E. coioides*, Ham.) which was severely rejected in fish market due to sever black discoloration of muscles. By parasitological examination of this rejected fish; the examination revealed the presence of *Huffmanella* spp eggs. The *Huffmanella* spp was identified according to the morphological keys recorded in *H. hamo* by Justine and Iwaki, 2014; *H. banning* (Moravec, 1987); *H. japonica* (Moravec et al., 1998); *H. lusitana* (Ramos et al. 2019) and *H. shikokuensis* (Moravec et al., 1998).

Our study revealed infection of muscles of Orange spotted grouper with *Huffmanella* spp. This identification was confirmed by the length and width of the eggs as 60–75 × 26–36 µm which nearly resembles to previous

study with Moravec et al. (1998) whose recorded *Huffmanella* spp. egg length as 58–69 × 26–30 µm. Egg shell in our study had protuberance as recorded by Moravec et al. (1998). Also; in our study, the eggshell envelop was smooth which revealed by scanning electron microscope; this finding was confirmed by Moravec et al. (1998).

Our study differentiated the species from other results in *H. huffmanii* which have spine on eggshell; these spines appear triangular as recorded in ultrastructure of transmission electron microscope by Žďárská et al. (2001), indicating that it is not *H. huffmanii*. In this study the protuberance appeared not regular as in *H. huffmanii* which appeared regular in shape and distribution. *H. huffmanii* recorded in swimbladder of freshwater fishes; Huffman and Moravec (1988), while our study recorded in muscles of marine fishes (Orange spotted grouper).

In the previous study on *H. japonica* by Moravec et al. (1998), accumulation of eggs in groups, in muscles of the infected goatfish, *U. bensasi* (Mullidae: Perciformes) were recorded. They found two stages of eggs (less advanced and advanced eggs).



**Fig. 3** **a** Cross section of muscles (m) and skin (sk) of *Epinephelus coioides* from fish market showing many black spots due to *Huffmanella* parasitism (p); **b** The blackish parasitic remnants (p) embedded in the myosepta of epaxial and hepaxial muscles, around vertebrae and vertebral spines; **c** Histopathological sections of the infected muscle of *Epinephelus coioides* with *Huffmanella* showing Multiple larvae (la) forming chronic focal granulomatous reaction associated with mononuclear inflammatory cells infiltration (ic) and surrounded with fibrous connective tissue proliferation (ct). (H&E stain)

There are 5 species of *Huffmanella* recorded to infect the muscles of marine water fishes *H. hamo* (Justine and Iwaki, 2014); *H. banning* (Moravec, 1987); *H. japonica* (Moravec et al., 1998); *H. lusitana* (Ramos et al. 2019) and *H. shikokuensis* (Moravec et al., 1998). The difference of these species according to the length of the eggs; eggshell and it's enveloped if present. The longest eggs of these species were *H. banningi* (99–108 × 42–45) with its shell spinose with minute canal on the envelope; and *H. hamo*; *H. lusitana* and *H. shikokuensis* were smooth shelled with smooth envelope; while *H. hamo* and *H. lusitana* were smooth shelled with no envelope.

From pathological point of view, in gross pathological examination, multifocal black parasitic remnants were noticed in the muscular tissue of the infected fish. This black discoloration is characteristic for *Huffmanella* in many fish species (Huffman and Moravec, 1988; Moravec et al., 1998; Bullard et al., 2012; Esteves et al., 2009; Esteves et al., 2016). In histopathological examination, inflammatory reaction evoked by the host tissue against the parasite was noticed. The inflammatory response appeared as chronic inflammatory reaction in the form of granuloma formation between the muscular tissue and it could be

agreeable with the findings of Moravec (1987) and Ramos et al. (2019) who recorded granulomatous reaction in some fish species infected with *Huffmanella* parasite.

## Conclusion

The current study highlights the morphological features and the associated pathological impact of *Huffmanella* spp infecting Orange-spotted Grouper (*E. coioides*) in Saudi Arabia. Although, the *Huffmanella* spp was recorded in only one reported case in orange spotted grouper, the severity of infection in the muscle caught our attention for the possibility of *E. coioides* role in dissemination of this parasite to other fish species including groupers' predators in marine ecosystem as sharks.

**Author's contributions** This study was conducted in cooperation between all authors. MA, MMA, Conceived and designed the study. MMI performed fish sampling and fish examination. MMA, examined and identified the eggs of the nematoda and photographed it; MAM, examined the histopathological samples and analyzed it. MA, MMA wrote the manuscript; all authors; drafting, revising and approved the final manuscript.

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**Data availability** All the authors declare that; all the data supporting the results reported in our article were found included in this article only.

## Declaration

**Conflict of interest** All authors declare that they have no conflict of interest.

**Ethical standards** All authors declare that the finding in this study was conducted on fish in markets. This article does not contain any studies with animals performed by any of the authors.

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