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NOTE

Important disease conditions of newly cultured species in intensive freshwater farms in Greece: first incidence of nodavirus infection in *Acipenser* sp.

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ABSTRACT: We describe here the main pathological conditions of freshwater fish recently introduced for intensive rearing (open ponds and recirculating freshwater systems) in Greece. Sturgeon were susceptible to skeletal abnormalities of the spine (scoliosis and lordosis) of unknown aetiology. Horizontal transmission of nodavirus from infected sea bass to sturgeon was detected for the first time. This caused serious pathology and clinical signs, such as lethargy and imbalance, leading to secondary infections with *Aeromonas hydrophila* and *Trichodina* sp. and chronic, but steady, mortality. Sea bass were very susceptible to nodavirus infection, monogenean infections and gas bubble disease. Mullet reared under recirculated and open-flow conditions were very sensitive to *Chilodonella* sp. infection, whereas catfish were susceptible to infection with *Ichthyophthirius* sp. leading to secondary infections with *A. hydrophila*, *Saprolegnia* sp. and Myxobacteria spp. Tilapia were very susceptible to gas bubble disease and *A. hydrophila*. This bacterium was associated with management manipulations for all species and fully responsive to corrective hygiene methods.

KEY WORDS: Freshwater aquaculture · *Acipenser* sp. · *Mugil* sp. · Recirculating systems · Nodavirus

INTRODUCTION

Aquaculture is one of the most important sectors of the Greek economy, due to the extensive shoreline and favourable climatic conditions. Greece has become the leading producer of marine fish such as sea bream *Sparus aurata* (L.) and bass *Dicentrarchus labrax* (L.) in the Mediterranean (FFI 1998). Recently, the lack of new sites for cage aquaculture and the ever increasing difficulties in obtaining licenses for new sites has increased interest in rearing euryhaline fish (such as sea bass and mullet) in freshwater land-based intensive systems (Dendrinos & Thorpe 1985). Preliminary reports on the growth performance of sea bass reared in freshwater in comparison to seawater have been encouraging (Klaoudatos et al. 1990). However, very limited information is available on the performance of sea bass and mullet in freshwater intensive conditions. Furthermore, new species for the region, such as *Acipenser gueldestaedi* and *Oreochromis* spp., have been introduced to a few freshwater farms, but these species are still under assessment and little is known about their growth performance and disease problems. The present paper describes the main pathological conditions observed in newly introduced freshwater species in Greece, some of them reared in intensive recirculating conditions. This is also the first report of nodavirus infection in *A. gueldestaedi* and the evidence of horizontal transmission of this virus in freshwater.

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MATERIALS AND METHODS

Fish. Sea bass *Dicentrarchus labrax* weighing approximately 50 to 80 g, reared in both recirculating and open freshwater production facilities situated in western Greece, showed nervous signs during an outbreak of clinical disease in late summer 2000 (during the present project period) when water temperatures were 25 to 28°C. The fish from both facilities were originally obtained from the same marine hatchery and transported to freshwater conditions when they weighed 1.5 g. They were subsequently reared in well freshwater in both recirculating and open-flow system facilities at a salinity of 0‰ and pH of 7.8. Sea bass were fed on commercial pellets specific to this species and their size.

Mullet *Mugil cephalus* was also reared in both recirculating and open freshwater production facilities. The fish from both facilities were originally obtained from the same marine hatchery and transported to freshwater conditions when they weighed 1.5 g. After transfer to the farm, all fish were reared in freshwater until the end of their production cycle. Mullet were fed on commercial pellets containing approx. 40 to 45% protein and 12 to 18% lipids, depending on fish size. Stocking densities for sea bass and mullet in the recirculating system was 45 kg m^{-3} and the temperature ranged from 18 to 21°C. In the open-flow system densities were 15 and 20 kg m^{-3} for sea bass and mullet, respectively. Water temperature ranged from 16 to 18°C in winter and 19 to 21°C in the summer (Table 1).

The other species examined in the present study were reared in freshwater in a commercial hatchery and transported to the farm when they weighed 2 to 5 g. All fish in the farm were reared in well water in an open-flow system at a salinity of 0‰ and pH of 7.8 throughout their production cycle. The production of each fish species and their particular rearing details are presented in Table 1. Sturgeon *Acipenser gueldestaedi* were fed on artificial pellets containing approx. 45 to 50% protein and 10 to 20% lipids depending on fish size. Tilapia *Oreochromis nilotica* were fed on commercial pellets produced for this species, and catfish *Silurus aristotelis* were fed on commercial pellets containing approx. 45 to 50% protein and 15 to 18% lipids, according to their size.

Necropsy and parasitological examination. During the period from 1997 to 2001, monthly samples of fish reared under intensive conditions were examined for diagnostic purposes. Macroscopic examination of the external surface of the gills, the total body and internal organs was conducted. Parasitological examination was carried out using methods described by Athanassopoulou (1990) and Bullock (1987). Identification of parasites was performed immediately after sampling with the keys of Yamaguti (1963) and Lom (1989). Parasite intensity was estimated as shown in Table 2.

Histopathological examination. Five percent of fish samples were examined histologically. Tissues from the internal organs, skin, gills and muscles were fixed in 10% buffered formalin. After decalcification, where needed, 5 µm histological sections were prepared and stained with the haematoxylin and eosin (H&E), Gram and Giemsa methods (Drury & Wallington 1980).

Bacteriological examination. Diseased fish showing clinical symptoms, as well as all fish with external lesions, were examined for the presence of bacteria. Kidney and spleen samples from diseased fish were inoculated onto Tryptone Soy Agar (TSA), according the methods described by Roberts & Shepherd (1997).

RNA extraction, reverse transcription and PCR amplification. Fresh organs (brain, eyes, liver, spleen, stomach, intestine, heart, swim bladder, kidney, gill) from diseased fish, particularly fish showing nervous clinical signs or eye lesions, were examined with

Table 1. Production details of freshwater fish reared in intensive systems. R = recirculating system, O = open-flow system

Species	Production (t yr^{-1})	System	Stocking density (kg m^{-3})	Recycling cycle	Monthly no. of fish examined	Total no. of fish examined
Dicentrarchus labrax	30	R	45	90%	30–40	820
		O	15	–		
Mugil cephalus	30	R	50	90%	10–15	300
		O	25	–		
Acipenser gueldestaedi					10–20	600
Meat	60	O	20	–		
Caviar	2–3 t per 6 yr cycle	O	20	–		
Silurus aristotelis	10	O	15	–	3–5	100
Oreochromis nilotica	10	O	15	–	3–5	100

Table 2. Estimation of parasite intensity

No. of parasites per viewing field (×40)	Intensity
1–2	+
3–4	++
5–6	+++
7–8	++++
>8	+++++

PCR for nodavirus. Ten mullets showing no apparent clinical signs, taken from the same recirculating system as infected sea bass, were also subjected to this examination. The RNA extractions were carried using the single-step method described by Chomczynski & Sacchi (1987) with TRIzol™ LS reagent (Gibco BRL). About 7.6 µl of the total RNA samples were subjected to reverse transcription using MMLV reverse transcriptase (Gibco BRL) followed by 30 cycles of PCR amplification using the primers F2 5′-CGTGTCAGTCATGTGTCGCT-3′ and R3 5′-CGAGTCAACACGGGTGAAGA-3′, designed to amplify the T4 region of the striped jack nervous necrosis virus (SJNNV) coat protein gene. The primer sequences and PCR conditions were the same as those described by Nishizawa et al. (1994). Ten µl of each PCR product were analysed by electrophoresis on a 2% agarose gel and stained with ethidium bromide (0.5 µg ml^{-1}). A 100 bp DNA ladder (Gibco, BRL) was run on the same gel to serve as a size marker. Positive controls were sea bass brain and eyes from nodavirus-infected marine farms, while in negative controls, RNA or cDNA were substituted with distilled water.

RESULTS AND DISCUSSION

The pathological conditions observed in the farmed fish are shown in Table 3.

Table 3. Pathological conditions of fish reared under intensive freshwater conditions

Condition	Species	Overall prevalence (%)	Location of infection	Production size (g)	Production system	Intensity
Bacterial and fungal diseases						
Aeromonas hydrophila and *Aeromonas caviae*	*A. gueldestaedi*	30	Internal organs, skin lesions	>100	Flow-through and earth ponds	++
	D. labrax	45	Skin lesions	>30	Recirculated	+++
	M. cephalus	5	Internal organs, skin lesions	5–100	Recirculated	+
Myxobacteria spp.	*M. cephalus*	3	Skin lesions	5–100	Recirculated	
	D. labrax	10	Gill lesions	>50	Recirculated	+
					and flow-through	++
Saprolegnia sp.	*A. gueldestaedi*	5	Skin lesions	All stages	All systems	+
	M. cephalus	5				+
	S. aristotelis	5				+
	D. labrax	5				+++
Viral infections						
Nodavirus	*A. gueldestaedi*	20	Brain	>500	Flow-through	++
	D. labrax	30	Brain, eyes	>50	Recirculated	+++++
					Flow-through	++
Parasitic infections						
Trichodina sp.	*A. gueldestaedi*	10	Gills, skin	All stages		
	M. cephalus	10	Gills, skin	All stages		
	D. labrax	10	Gills, skin	All stages		
Ichthyophthirius sp.	*S. aristotelis*	80	Gills, skin	All stages	Flow-through	+++++
	D. labrax	15	Gills, skin	All stages	Recirculated	++
	O. nilotica	10				
Chilodonella sp.	*M. cephalus*	80	Gills, skin	All stages	Flow-through	+++++
					Recirculated	++
Diplectanum aequans	*D. labrax*	80	Gills	>30	Flow-through	+++++
Others						
Skeletal deformities	*A. gueldestaedi*	40	Skeleton	>500	Flow-through	+++++
Gas bubble disease	*D. labrax*	30	Skin, gills	All stages	Flow-through and recirculated	+++
	O. nilotica	20	Eyes, gills	All stages	Flow-through	++

Dicentrarchus labrax

These fish showed very limited mortality following transfer to freshwater, although *Aeromonas hydrophila* infections occurred in both facilities which were successfully treated with antibiotics. The outbreak of nodavirus occurred approximately 8 mo after transfer of the fish to freshwater. During this outbreak, which lasted approximately 1 mo, the clinical signs in fish kept under recirculating facilities included abnormal swimming behaviour (erratic circling), blindness due to lesions in the eyes, emaciation, dark colour, skin abrasions and losses reaching a total mortality of 30% per day. Skin lesions were often infected secondarily by *Saprolegnia* sp. In the open-flow facility, fish showed no nervous clinical signs and only eye lesions were present with low mortality (less than 5%). The presence of the virus in the traumatic lesions apparent on the head and in the eyes was constantly confirmed by PCR. At the time of the outbreak, the water temperature was 28°C. Histologically, lesions typical of viral nervous necrosis virus (VNNV) infection were observed in the brain, spinal cord and in the eyes of all diseased fish, as described in detail by Athanassopoulou et al. (2003).

These sea bass were possibly first infected when they were introduced to the virus at the marine hatchery, and were asymptomatic carriers during their earlier life in freshwater. The virus has been isolated from cultured, asymptomatic sea bream carriers (Castri et al. 2001) and recently in tropical fish (Hedge et al. 2003). Nodavirus stored in freshwater under laboratory conditions has been shown to be less stable than that in seawater, and no viable virus could be detected after 6 mo storage (Frerichs et al. 2000). Sea bass developed clinical signs of the disease after over 8 mo in freshwater, possibly demonstrating that even asymptomatic carriers can develop clinical disease in real farm situations (Athanassopoulou et al. 2003).

Sea bass was also found to be very susceptible to infection with the monogenean parasite *Diplectanum aequans* in the gills, especially in colder months. This condition caused high mortality and was related to a particular batch of introduced fish, reared only in open-flow systems. Another important problem of this species was its sensitivity to gas bubble disease caused by hydrogen sulphite and CO_2 (Athanassopoulou et al. 2003). Sea bass either infected with nodavirus or exposed to high gas levels in the water presented traumatic lesions due to the blindness and irritation caused by these conditions, complicated by secondary fungal and myxobacterial infections.

Acipenser gueldestaedi

Diseases of cultured sturgeon are reported mainly from North America (Hedrick et al. 2001). It is a very desirable fish for rearing because of the fast growth rate, good digestion of food, even with no specific diets, and the ability to be cultured under different systems (Williot et al. 1993, Paschos et al. 1998). Furthermore, it has a wide temperature tolerance, is very tolerant to stress, and the mortality after 5 cm is less than 5%. Under Greek farming conditions, the weight gain is between 70 and 110 g in 180 d (Paschos et al. 1998) whereas in Portugal, a gain of 900 g in 9 mo has been reported (Lopez-Rosario 1991).

In our study, *Acipenser gueldestaedi* was found to be very susceptible to both nodavirus (Fig. 1) and spinal deformities beyond a weight of 500 g. After reaching this weight, fish in open-flow systems started to show skeletal abnormalities with increasing prevalence. Histopathology performed both in Greece and Canada was inconclusive as to the aetiology of these skeletal deformities. They could not be proved to be associated with the feed. Similar deformities have been observed before in cultured sturgeon (Koksal et al. 1999), but have not yet been fully investigated. At a later stage, fish could not swim properly, could not feed and soon were prone to secondary infections with *Aeromonas hydrophila*. Several other fish, which were healthy and well developed with no skeletal deformities, presented lethargy, turned upside down in the tanks and seemed dead. However, they responded well to stimulus and survived for a long time, although unable to eat. No parasites or microbial infections were found in these fish.

In PCR analysis, a 426 bp amplicon was constantly detected from nucleic acid preparations of brain tissue

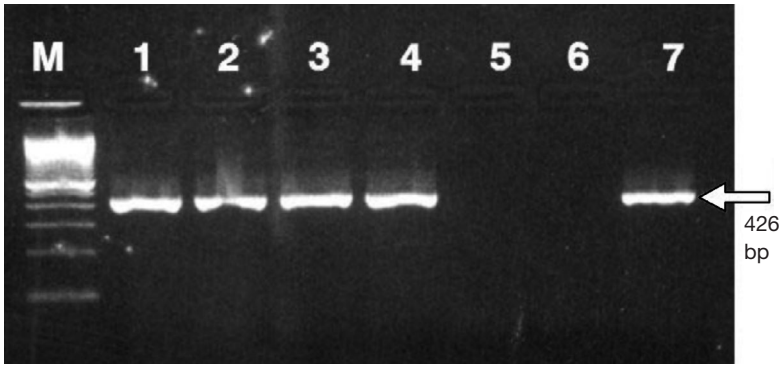

Fig. 1. *Dicentrarchus labrax* and *Acipenser gueldestaedi*. RT-PCR products specific for coat protein of SJNNV. Lane M: 100 bp DNA ladder (Gibco-BRL), Lanes 1 and 2: sea bass eyes from freshwater open system facilities; Lane 3: sturgeon brain from freshwater open system facilities; Lane 4: sea bass eyes from freshwater recirculating facility; Lanes 5 and 6: negative controls; Lane 7: positive control

from sturgeon presenting all the above symptoms. This was comparable with nodavirus-positive samples from marine farms (positive control, Fig. 1) as well as a positive sea bass sample from the same freshwater farm reported recently (Athanassopoulou et al. 2003). In this study, nodavirus infection of sea bass in freshwater caused neurological signs and histopathological lesions similar to those observed in the marine environment, but with lower mortality.

In contrast to sea bass, in which nodavirus infection was found in both brain and eyes, infection was present only in the brain of sturgeon. Affected brain showed pathological changes in the mesencephalon, medulla oblongata and cerebellum. These lesions in the brain and the spinal cord included vacuolation that was most conspicuous in the grey matter (Fig. 2). A variable degree of gliosis was seen in a few cases, in both vacuolated and non-vacuolated areas of the central nervous system. These lesions were similar to those observed in brains of infected sea bass.

Preliminary sequencing results show a close link between sea bass and sturgeon nodavirus (authors' unpubl. data). This indicates that the virus has passed horizontally in the farm from sea bass to sturgeon. Although there was no water contact between these fish, contamination may have occurred via equipment. The presence of this virus in *Acipenser gueldestaedti*, reared totally in freshwater in the same farm as infected sea bass, demonstrates its ability not only to survive but also to multiply in freshwater and indicates that, under the selective pressure related to crossing the species barrier, nodavirus remained stable. This is the first time that this has been reported in sturgeon and for a freshwater environment.

Fig. 2. Nodavirus infection in *Acipenser gueldestaedi*. Medulla oblongata where numerous vacuoles are observed. H&E, ×400

Mugil cephalus

This species was very tolerant to intensive rearing and performed very well throughout the production. Water was reused in the recirculating system and the fish were in contact with infected sea bass before, during and after the nodavirus outbreak. However, this species did not develop any signs of nodavirus infection and PCR tests were negative. The only problem observed was recurrent infections with *Chilodonella* sp. which were present at high intensity throughout the year. No other species in the farm was found to be infected with this parasite, not even sea bass in the same recirculating system, which suggests that it is host-specific. Infection with this parasite was very difficult to treat in mullets.

Silurus aristotelis

This species was introduced experimentally into the farm for a short time and did not do well in terms of acceptable growth rate and disease tolerance. *Ichthyophthirius* sp. infections caused high mortalities. The parasite was initially found in the gills and then spread to the adjacent skin, resulting in big erythematous lesions These were secondarily infected by *Saprolegnia* sp. The treatment of this condition in the catfish was very difficult. The intensity was high, especially in the summer. Another problem with *Silurus aristotelis* was its susceptibility to hydrogen sulphide present in the water at levels of 0.001 to 0.04 and 0.01 to 0.02 mg l^{-1} in the cold and warm months of the year, respectively. The prevalence and intensity of symptoms were constant throughout the year. These levels of hydrogen sulphide are higher than the <0.001 mg l^{-1} toxicity limit referred to in the literature (Ghittino 1983) and may have been the primary obstacle to the successful rearing of this species under intensive farming conditions (Martin 1985). However, more research is needed to establish this speculation.

Oreochromis nilotica

Although several diseases have been reported in cultured tilapias, such as *Chilodonella* sp. infections (Hussain 1984, Sagua 1987), in the present study, this species proved to be very tolerant to diseases. The only condition found was *Ichthyophthirius* sp. infection in the gills at low density during summer and gas bubble disease in the gills and eyes, but this was quickly resolved by mechanical treatment of the water. The parasite infection was successfully treated and no other problems were present.

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