Occurrence of diseases in fish used for experimental research

(Article begins on next page)

17 September 2024

1 **Occurrence of diseases in fish used for experimental research**

2

3

4 **Carmelo Iaria[1], Concetta Saoca[2], Maria Cristina Guerrera[2], Sara Ciulli[3],**

5 **Maria Violetta Brundo[4], Giuseppe Piccione[2], Giovanni Lanteri[2]***

6

7 [1]Department of Chemical, Biological, Pharmaceutical and Environmental

8 Sciences, University of Messina, Italy.

9 [2]Department of Veterinary Sciences, Experimental Ichthyopathology Center

10 of Sicily, University of Messina, Italy.

11 [3]Department of Veterinary Medical Sciences, University of Bologna, Italy.

12 [4]Department of Biological, Geological and Environmental Sciences,

13 University of Catania, Italy.

14

15

16 ***Corresponding author**: Prof. Giovanni Lanteri, Department of Veterinary

17 Sciences, Polo Universitario Annunziata, University of Messina, 98168

18 Messina, Italy. Tel: +39 3503707. Fax: +39 0903503975. email:

19 glanteri@unime.it

20

Abstract

21

22 The objective of the present study was to evaluate the occurrence of

23 pathogens and diseases in laboratory fish, over a 10-year period, at the Centre

24 for Experimental Fish Pathology of Sicily (CISS), University of Messina. In

25 addition, we aimed to highlight the subclinical effects of these diseases on

26 research endpoints, the importance of animal health and welfare issue of

27 organisms that cause clinical disease and that could directly change the results

28 or increase the variability or a lack of reproducibility of experiments. For this

29 purpose, 411 diseased fish of different species, out of a total of 2820, and

30 belonging to four marine species (*Dicentrarchus labrax*, *Sparus aurata*,

31 *Argyrosomus regius* and *Mugil cephalus*) and to four fresh water species

32 (*Danio rerio*, *Carassius auratus*, *Xiphophorus variatus* and *Poecilia*

33 *reticulata*) were examined in this study. Our results showed that

34 mycobacteriosis and myxosporidiosis were the most important diseases found

35 in our research fish and the results represent a useful tool to obtain wider

36 knowledge on the incidence of various diseases in different fish species.

37 Further studies in this field are necessary to improve knowledge on the state

38 of health state of fish used for research.

39

40 **Key words**: experimental research, fish diseases, pathological conditions,

41 health monitoring.

42

Introduction

Several pathogenic microorganisms are responsible for clinical diseases in laboratory animal species and can influence the research by confounding experimental results. For this reason, a constant check-up of animals used for research is necessary to maintain an optimal state of health in laboratory animals.[1]

It is well known that many infectious agents harm welfare status or reduce breeding performances of animals such as rabbit and rodents, which are commonly used in laboratory experiments.[2]

In the last few years, a field that is arousing growing interest is the use of fish as experimental animals for biomedical research, both for human research and for aquaculture purposes. However, little is known on the pathogens of fish compared to mice, rats and rabbits; information regarding pathological surveys or the health status of fish used in experimental trials is scarce. This is probably due to the fact that, despite an increasing use of a wide range of species of fish in several research projects, no particular attention has been paid to health management in the field of laboratory animals: with regard to the use of fish there is not even a suitable pathogen control at fish facilities.[3–8] This aspect, which has unfortunately been neglected, could be a useful tool to control the biological and experimental variability of fish used in research procedures, providing useful information for a correct interpretation of results.[9] However, given that pathogens can affect fish in a very similar

3

65   manner to rodents, current regulations are intent on improving health

66   management in fish research facilities.

67   To date, only few studies regarding the prevalence and impact of some

68   microorganisms on animal health status have been conducted.[10–14]

69   Spagnoli et al.[15,16] studied the effects of *Pseudoloma neurophilia*, one of the

70   most serious pathogens in zebrafish facilities, on the nervous system, and its

71   implications for neuro-behavioral research.

72   Previous studies were also conducted in 2009 by Broussard et al.,[11] Hegedus

73   et al.[17] and van der Sar et al.[18] on *Micobacterium marinum*. It is known that

74   mycobacterial infections, frequently isolated from laboratory fish,

75   significantly affect zebrafish health status and transcriptome response.[19,20] On

76   the contrary, only sporadic date are available on pathogens in other species of

77   laboratory fish.

78   Today, both the use of specific pathogen free (SPF) animals for experimental

79   protocols and the application of appropriate pathogen control strategies

80   should be essential conditions in the field of fish research. Currently, other

81   fish species used for research experimentation come mainly from the

82   aquaculture sector, and the absence of pathogens in their traded fish lines,

83   such as zebrafish, cannot be guaranteed.[21–23] This said, a more detailed study

84   of prevalence and distribution of microorganisms within fish research

85   facilities is necessary to enhance pathogen control strategies, preventing entry

86   and spread of such noxious agents. Furthermore, the effects on anatomy and

87　physiology of research fish caused by the presence of an infectious agent must

88　be carefully evaluated, improving experimental result predictivity and

89　reducing the number of animals which should be used.

90　The aim of this study is to provide an overview on prevalence, clinical

91　presentation and pathological findings of the main diseases encountered at the

92　CISS laboratory. Our research offers a valuable contribution to knowledge

93　about the prevalence and the effects of different fish disease processes on

94　research.[13]

95

Materials and Methods

The facility

This study was carried out at the University of Messina, CISS establishment. The laboratory has been recognized by the Italian Ministry of Health since 2006 and was authorized as an establishment for the production of fish for experimental purposes in 2010. The CISS facility has 3 rooms: a separate quarantine room, a zebrafish breeding room and a main room, in which all experiments are carried out. The main room is equipped with 50 fiberglass and plastic tanks with a capacity of 120 to 800 liters, each one provided with independent biological-sand and activated carbon filters, aerator pump and automated monitoring system for continuous water parameter controls (Oxywifi2, Tecnos, Chioggia, Italy). Zebrafish maintained in the zebrafish breeding room are reared, in a ZebTEC Active Blue Stand Alone system, Tecniplast. In these three recirculating housing systems, the water is sterilized with UV (minimum dose 135,000 $\mu Wsec/cm^2$) and derives from reverse osmosis-treated city water to which is added salt to a conductivity of 450 µS. Environmental conditions at the primary enclosure (water tanks) are maintained at 27± 1 °C temperature values, pH 7.2 ± 0.3, dissolved oxygen content (DO) of 6.00 ppm for freshwater species. Marine species are held in seawater, artificially reconstituted by the addition of salt (Blue Treasure Sea Salt, Qingdao Sea-Salt Aquarium Tecnology Co., Ltd, China), and in which the following

118   parameters are guaranteed: temperature of 20-22°C, pH 8, DO 7 ppm and

119   salinity of 1035. Moreover, freshwater and marine fish are exposed to a

120   light/dark cycle (14L/10D) and fed twice daily with Artemia Nauplii (JBL

121   Artemio Pur, BL GmbH & Co. KG, Dieselstraße 3, 67141 Neuhofen,

122   Germania) in the morning, and a commercial diet (GEMMA, Skretting

123   Australia, 26 Maxwells Road, Cambridge, Tasmania) in the afternoon.

124   Wild-type zebrafish and other freshwater and marine species are often

125   procured for outcrossing or experimental purposes from several sources with

126   pathogen and disease status. The health status of all fish housed in CISS is

127   checked daily through clinical observations of animals and histopathological

128   evaluation of retired stocks (older than 18 months for zebrafish) or periodical

129   dead fish. A fish sentinel monitoring evaluation is carried out every six

130   months   through   bacteriological,   parasitological,   molecular   and

131   immunohistochemical screenings of pre-filtration and post-filtration sentinel

132   specimens for the zebrafish facility. Fish species acquired from the

133   aquaculture industry or ornamental fish trade or animals used for

134   experimental purposes, without proper health certification are examined, after

135   determination of a realistic sample size from the fish stock, by means of

136   aforementioned lethal diagnostic tests, according to population size and

137   researchers' needs, during quarantine period or at the end of the trials.[24]

138   *Animals*

7

139   Our study was carried out on a total of 2820 fish including 1661 freshwater

140   fish from six different species (1465 zebrafish *Danio rerio*, 16 goldfish

141   *Carassius auratus*, 30 variable platyfish *Xiphophorus variatu*, 70 guppies

142   *Poecilia reticulata*, 60 European carp *Cyprinus carpio*, 20 tench *Tinca tinca*),

143   and 1159 marine fish from seven other species (296 European seabass

144   *Dicentrarchus labrax*, 323 gilthead sea bream *Sparus aurata*, 80 meagre

145   *Argyrosomus regius*, 340 mullet *Mugil cephalus*, 20 bogue *Boops boops*, 20

146   European eel *Anguilla anguilla* and 80 dentex *Dentex dentex*) (Table 1). From

147   September 2007 to June 2016, all these fish, maintained under controlled

148   conditions at CISS and involved in experimental projects in many different

149   fields of research, were examined by the diagnostic service of CISS and

150   screened for pathogens using fresh sample microscopy, histology techniques,

151   culture methods and PCR. All experimentation procedures have been

152   approved by the Animal Welfare Committee of the University of Messina

153   (O.P.B.A.), the National Institute of Health and the Italian Ministry of Health

154   according to previous laws, Directive 86/609/CEE, legislative decree 116/92,

155   the laws currently in force, EU Directive 63/2010 and Legislative Decree

156   26/2014.

157   *Experimental procedures*

158   A complete necropsy was performed on all specimens included in the surveys

159   (moribund fish, animals which died within the previous 12 hours and live fish

160   euthanized during or at the end of the studies).

After decontamination of the external surface with 70% ethanol, carcasses were analysed to highlight gross changes. Microscopic examination of smears from skin, gills, blood and internal organs (intestine, liver, gall bladder, gonads, kidney, and hearth) was done; specimens were also processed for histological evaluation, cultural and molecular assays. All biological samples were collected aseptically.

For blood sampling, imaging and/or experimental procedures, live fish were anesthetized with MS-222 (Sigma Aldrich, Milano, Italy) using 200mg/L (Buffered with 0.4 mg/L of sodium bicarbonate).[25] Exposure to anaesthetic solution varies according to the aim of the study as well as to the fish species.[26] In some cases, after surgery, antibiotic therapy was administered with enrofloxacin (0.16 ml/L) dissolved in water. Fish euthanasia was carried out by means of chemical method, using MS-222 overdose (500mg/L) in all adult fish, or hypothermic shock in ice bath (5-parts ice to 1-part system water at a constant temperature of 0 °C) in zebrafish, and no adjuvant method was used; adults were exposed for at least 10 min after cessation of opercula movements, and fry 4 to 7 dpf at least 20 min after cessation.[27] Small samples from all organs and tissues analysed were subjected to histopathological and immunohistochemical examinations.

For histopathology, samples from all organs and tissues, or entire fish body for zebrafish and similar fish size, were fixed in 10% neutral buffered formalin for 72 h following routine methods. Only in the presence of calcified

9

183   tissues were samples decalcified using Electrolytic Decalcifier Bio-Optica for

184   some hours (3h to 6h) in relation to size, dehydrated in series of graded

185   alcohol and then embedded in paraffin. Wax tissue samples were rinsed in tap

186   water, dehydrated by rinsing in alcoholic solution, clarified in xylene and

187   finally embedded in paraffin wax. Five µm thick tissue sections were stained

188   with Hematoxylin-Eosin, Masson's Trichrome, Alcian Blue Pas, Ziehl

189   Neelsen, Grocott and photographed under a light microscope Zeiss axiophot.

190   For immunohistochemistry, samples of organs and tissue were formalin fixed

191   and processed for paraffin embedding. The blocks were cut into 10 μm thick

192   sections, mounted on gelatine-coated microscope slides, deparaffinised,

193   dehydrated and processed for indirect peroxidase immunohistochemistry, as

194   described in Marino et al.[28]

195   Antibodies used were rabbit polyclonal antibody against S-100 (Dako,

196   Glostrup, Denmark, diluted 1:1000), vimentin (clone Vim 3B4, Boehringer-

197   Mannheim, Germany, diluted 5 µg ml^{-1}) and calretinin (Chemicon, Temecula,

198   Ca, USA, diluted 1:500).

199   A polyclonal antibody against *Mycobacterium avium* (NeoMed, Casorezzo,

200   Italy, diluted 1:100) was used to recognize the genus.

201   In some cases, a bacteriological examination was carried out. On isolated

202   microorganisms, biochemical tests were performed to identify the species.

203   For the bacteriological examination, liver and kidney samples were collected.

204   Tissue samples were cultivated on the following media: brain heart infusion

205   (BHI) and brain heart infusion agar (BHIA) + 1.5% NaCl, Marine Agar 2216

206   E, Blood Agar + 1.5% NaCl, thiosulfate citrate bile salts sucrose TCBS +

207   1.5% NaCl and incubated at 24° C x 24-48 h. The following tests were

208   performed on all the isolates: growth at different salinity and temperature,

209   Gram stain and strain identification by the miniaturized system, API 20E. The

210   Bionor Elisa kit specific against *Photobacterium. d.* subsp. *p.* was used to

211   confirm the results.

212   Regarding biomolecular analysis, DNA was investigated on wax embedded

213   tissue samples prepared for histological examination. DNA was extracted by

214   using the *Gene Elute kit* (Sigma Chemical). PCR was targeted to the internal

215   transcribed spacer (ITS), using universal primers. To detect specific DNA

216   referable to the genus *Mycobacterium,* the primers Int1 and Ext2 (5'-

217   CCCCATCGACCTACTACG-3';   5'-CCCGGACAGGCCGAGTTT-3')

218   were used. PCR products were analysed by gel electrophoresis and sequenced

219   by Applied Biosystems 3.1 version kit and 3130 genetic analyser Applied

220   Biosystems 3130. The sequence data collected were compared with known

221   sequences in Genebank, by WU BLAST 2 software.

222   *Limphocystivirus* infection was confirmed by a molecular assay. Viral DNA

223   was detected, extracted by cutaneous tissue and quantified by a real time PCR.

224   On cadaver specimens, radiographic examinations were performed in

225   zebrafish and goldfish. Standard radiograph equipment used for small animal

226   patients is suitable for fish radiography. Radiographs were obtained using

227 exposure settings of 40 kV and 4 mAs for fish ranging from 9 to 14 cm length

228 and 40 kV and 6.5 mAs for fish between 17.7 and 25.5 cm in length. Fish

229 examined were radiographed in three projections in the following order:

230 ventral-dorsal, 30° right lateral-dorsal-left lateral-ventral oblique and 30° left

231 lateral-dorsal-right lateral-ventral oblique.

232 **Results**

233 Both clinical and subclinical spontaneous diseases found in each species

234 over 10 years of activity at the CISS facility are listed and summarized in

235 Table 1.

236 *Danio rerio*

237 Twenty-four adult zebrafish from transgenic lines farmed at CISS showed

238 different congenital abnormalities (craniosychosis attributable to incomplete

239 bone welding of the cranial vault, albinism, microcephaly associated with

240 microphthalmia)

241 Several zebrafish embryos showed numerous skeletal abnormalities, such as

242 the presence of vertebral axis abnormalities and pectoral and caudal fins

243 anomalies.

244 Eight adult male, wild-type zebrafish were affected by *Mycobacteriosis* and

245 showed serious emaciation and moderate ascites. All specimens were

246 euthanized and processed for histological, immunohistochemical,

247 bacteriological and molecular examinations.

248 Organs and tissues showed no macroscopic changes. Histological section

249 confirmed a classic systemic granulomatosis; several granulomas were

250 located on the parenchyma of spleen.

251 Microbiological and molecular examinations confirmed the presence of

252 *Mycobacterium* and showed other bacterial species such as *M. marinum*, *M.*

253 *chelonae* and *M. fortuitum* in examined tissue.

254 Four adult female, wild-type pond zebrafish, imported from Singapore and

255 acquired for reproduction purposes from the ornamental fish trade before the

256 new EU Directive 63/10, were found positive for cartilaginous cysts in the

257 gills at histological examination; on the basis of morphological criteria,

258 parasites were identified as trematode belonging to the genus *Centrocestu*s

259 sp. (Figure 1).[29]

260 One adult wild-type *D. rerio* showed a black mass located on the under-lip

261 margin. Histological evaluation and immunohistochemistry were carried out

262 and a schwannoma, whose description was reported by Marino et al.[30], was

263 diagnosed.

264 *Carassius auratus*

265 A physic-chemical test revealed a concentration of ammonia in the water over

266 the normal level (0.5 mg/L) in 15 goldfish (9 males and 6 females) that

267 showed symptoms of intoxication (erosions and epidermal bleeding). The

268 fish-tank water was partially substituted, and levels brought back to normal

269 in 24 h.

270 A goldfish showing an abdominal distension was subjected to radiographic

271 examination that confirmed the presence of renal cysts.

272 In 4 goldfish (1 male and 3 females) that showed white, dorsal masses, a

273 cytological examination was carried out after anaesthesia. Myxosporidiosis

274 was diagnosed, and the genus *Myxobolus* identified (Figure 2). The fish were

275 euthanized after the identification of the parasitic spore.

276 Schwannoma was found in three, adult, seven-year-old goldfish (1 male and

277 2 females) (Figure 3); these tumours appeared as subcutaneous soft nodular

278 bulges which in HE stained sections showed defined borders and were

279 composed of elongated cells.

280 Five adult *C. auratus* (3 males and 2 females) reared as broodstock in the

281 same tank, 2 *Xiphophorus variatus* acquired from a private aquarium shop,

282 and 14 *P. reticulata* (10 males and 4 females) reared in the same tanks in the

283 facility and used for fish production within CISS, developed a chronic

284 infectious disease, with ulcerated skin lesions and tumour-like nodules. The

285 specimens of *C. auratus* and *P. reticulata* were euthanized, while those of *X.*

286 *variatus* died spontaneously. All these fish were found positive for

287 mycobacteriosis after histological examination that showed granulomatosis;

288 Ziehl-Neelsen staining demonstrated the presence of *Mycobacterium*.

289 *Dicentrarchus labrax*

290 The dinoflagellate *Amyloodinium* sp. was identified on fresh skin and gill

291 samples analysed in two fish for parasitological examination at post-mortem

292 examination.

293 Twenty-five fish died of chronic photobacteriosis showing necrotic foci and

294 granulomas in spleen (Figure 4), liver, kidney and heart due to the presence

295 of *Photobacterium damselae* subsp. *piscicida*.

296 Fifty-eight *D. labrax* were found positive for sphaerosporosis (*Sphaerospora*

297 *dicentrarchi*) in intestine at post- mortem histopathological examination.

298 Eight sea bass that showed signs of acute infectious disease with skin and

299 multi-organ haemorrhages were euthanized. These fish were positive for

300 vibriosis (*Vibrio anguillarum*) from cultural examinations.

301 *Sparus aurata*

302 After some months of farming, 32 apparently healthy four-month-old *S.*

303 *aurata* juveniles, (both males and females), showed the typical clinical signs

304 of lymphocystis virus disease with the appearance of several skin

305 proliferations on the fins, mouth, dorsum and on the lateral side of the body.

306 Three fish per tank were euthanized and autopsied; tissue samples were

307 collected for histopathological and molecular analyses that confirmed the

308 lymphocystis disease (Figure 5) and the presence of viral DNA in all analysed

309 fish.

310 One single case of 'winter disease' was registered in a gilthead sea bream

311 acquired a few days after the transfer from a fish farm in early February. The

312   fish showed pale skin, clouding eye, abdominal swelling, pale liver, ascites

313   and haemorrhagic gut. The sea water on the fish farm was 14 °C. Twenty-

314   three *S. aurata* (10 males and 13 females) were found histologically positive

315   for *enteromyxidiosis* at post mortem examination, and *Enteromyxum leei* was

316   identified.

317   Seventy adult fish (32 males and 38 females) were positive at parasitological

318   exam for gall bladder myxosporidian infection; *Ceratomyxa sparusaurati*

319   was detected in fresh samples of the gallbladder of these fish, which no

320   showed clinical signs. Myxosporidian parasites had colonized the mucosa of

321   intestine with different severity.

322   *Cryptocarion irritans* was seen in 25 fish after a partial water substitution

323   with marine water. Mortalities appeared three days after the change and the

324   only obvious macroscopic signs were some white spots seen on skin of

325   moribund fish.

326   *Argyrosomus regius*

327   Forty adult *A. regius*, both males and females, at histological examination,

328   showed a chronic evolution of the granulomas localized in spleen, liver,

329   kidney, intestine and heart. Six of these subjects died later of severe

330   cryptocarioniasis.

331   *Mugil cephalus*

332   Sixty-four wild adult *M. cephalus* (38 males and 26 females) were found

333   positive for zoonotic pathogens. No clinical signs were registered although

334 four were histologically positive for trematode metacercaria of the genus

335 *Heterophyes* and 60 were demonstrated positive for mycobacteriosis.

336 The genus *Mycobacterium* was confirmed by means of

337 immunohistochemistry and cultural exams, whereas PCR confirmed that the

338 main species was *M. fortuitum*. Thirty-two adult mullet showed lymphocystic

339 diseases.

340 The other fish species *(Dentex dentex*, *Cyprinus carpio*, *Boops boops*, *Tinca*

341 *tinca and Anguilla anguilla)* did not show any disease.

342

Discussion

In this study, the authors deal with diseases found both in zebrafish and other fresh and salt water teleost, providing useful data for research.

Zebrafish (*D. rerio*) is the principal teleost species used at CISS. This fish can suffer from a wide range of pathologies. Congenital abnormalities, for example, are relatively common in *D. rerio* and some zebrafish strains may be more prone to develop morphological deformities which are compatible or not with life.

In the last few years, the use of zebrafish has increased considerably in many fields of research. Low breeding costs, high prolificity of the broodstock, rapid organogenesis, transparency of embryos and the extensively studied development process have all made the zebrafish a valuable animal model to study organogenesis disorders, test novel therapies and screen toxicity of chemicals and potential teratogens.[31] In particular, zebrafish embryos provide a reliable model for the high-throughput screenings of chemicals as they present the advantages of an in vivo model and the convenience of an in vitro approach.[32,33] Recently, the use of zebrafish for development toxicology assays has been encouraged given the high concordance with animal data that has been demonstrated.[34–37] Zebrafish developmental toxicity assays (ZEDTA) have a good predictive value regarding assessing chemicals or candidate drugs for human safety. However, the use of various zebrafish lines can determine data discrepancy among different laboratories and with

365 mammalian in vivo results as genetic background differences may affect

366 zebrafish strain susceptibility to a particular test substance. Therefore, in

367 some cases, this variable could produce false positive or false negative

368 results.[38] For correct interpretation of results, not only the quality of used

369 clutches but also the rates and the kind of variation in morphology

370 encountered in control groups in historical controls of the used zebrafish

371 strain should be considered. However, in most cases, all this information is

372 rarely provided. Therefore, in light of the growing use of zebrafish for

373 teratologic or toxicity screenings, we need to expand our knowledge on the

374 spontaneous malformation rates in any of the generally used wild-type,

375 transgenic and mutant lines.

376 In our study, the main problem was mycobacteriosis, which represents a

377 potential risk for personnel. Numerous studies have previously been

378 conducted on mycobacteriosis in zebrafish.[39–44] Instead, cartilaginous cysts

379 due to *Centrocestus* sp. metacercariae, despite being a potentially dangerous

380 zoonosis, are not a health risk for laboratory personnel as the transmission of

381 parasites occurs by ingestion of raw fish. Regarding congenital abnormalities

382 that are relatively common in zebrafish, they can probably be exacerbated by

383 transgenesis. The knowledge of ontogenetic anomaly prevalence in the

384 different zebrafish lines is extremely important in trying to achieve high

385 health standards and more reliable results. Although *Pseudoloma neurophilia*

386 is the most common pathogen detected in zebrafish husbandry coming from

387 our facility, histological and molecular examinations did not show any

388 evidence of this pathogen in either the cut brain or spinal cord tissue.[45]

389 European sea bass coming from fish farms, can develop vibriosis and

390 photobacteriosis. These two infectious diseases may interfere on experiment

391 feasibility causing a high rate of mortality (90-100% in acute forms).

392 Sphaerosporosis is a parasitic disease due to the myxozoa *S. dicentrarchi*.

393 These protozoa can be endemic in some fish farms; it is an opportunistic

394 pathogen, generally with low pathogenicity that can sometimes cause severe

395 tissue changes and death. In our study, 58 *D. labrax* out of a total of 296

396 showed few myxosporeans found in the *muscularis mucosae* of the intestine

397 without any signs of inflammation.

398 Regarding the dinoflagellate *A. ocellatum* that we found in two specimens of

399 *D. labrax*, the low parasitic charge, low incidence of disease and low tissue

400 damage observed in the gills did not interfere with results of the trial.

401 The protozoans *C. irritans*, *E. leei* and *Ceratomyxa* sp. that were found in

402 some *S. aurata* fish during our study are frequently detectable in this species.

403 *C. irritans* under experimental conditions can be lethal, with a very high

404 percentage of mortality and thus must be immediately identified and excluded

405 from experimental trials. *E. leei* and *Ceratomyxa* sp usually show low

406 pathogenicity for gilthead sea bream.

407 'Winter disease' is a metabolic syndrome common in captive sea bream

408 during the cold season; it causes gut dilation filled with undigested food and

409 necrotic-haemorrhagic debris, which can sometimes be lethal[46,47]. Another

410 disease commonly detected in *S. aurata* is lymphocystis disease that, although

411 a self-limiting viral disorder, may compromise the immune system[48].

412 Non-infectious systemic granulomatosis (GSNI), found in 40 *A. regius* of our

413 experiment, is a nutritional disorder which is often detectable in coelomic

414 organs of meagre less than 2 years in age. It is not transmissible and never

415 causes death. No pathological agents have been related to this disease. It has

416 been suggested that these granulomatous changes are caused by the

417 deposition of tyrosine crystals related to a nutritional deficiency, possibility

418 due to poly-hypovitaminosis[49].

419 The results of our study showed that mycobacteriosis and myxosporidiosis

420 are the main risks in experimental fish facilities. These two diseases must be

421 prevented and mitigated as they can cause mortality and cannot effectively be

422 treated. For this purpose, particular care should be applied when new fish are

423 introduced into a facility, respecting a quarantine period for mycobacteriosis

424 (at least 30 days)[50] and checking the fish for pathogens by in vivo methods

425 (often hard to apply in adult zebrafish), or sacrificing a statistically significant

426 number of fish for histopathology and molecular diagnostic. Production of fry

427 and alevins within the facility permit to avoid the above-mentioned risk of

428 transmission of infectious disease via egg bleaching. Most of the diseases

429 encountered in our experience could cause failure of experimental trials, or

430   the necessity to exclude specimens from statistical evaluation of results; this

431   represents a damage in terms of costs and time for researchers.

432   To provide a total picture of research results, it is important to report the

433   health and welfare status of the fish, including programs and methods applied

434   to detect pathogens, agents to be investigated, methods of registration, report

435   of findings and interpretation of the results. Obviously, the availability of

436   historical data on the health status of the spawners and alevins possesses a

437   definitive, strong value.

438   The diagnosis of disease in fish is sometimes difficult due to the presence of

439   disorders with unknown cause and whose diagnosis is often limited to

440   morphological evaluation only. In some cases, to detect specific pathogens,

441   an ultra-structural exam must be carried out, although this technique has low

442   reliability and needs a very long time, often not compatible with the needs of

443   the facility.[51] A sound knowledge of the pathogenesis of diseases could

444   provide an improvement in detection techniques for pathogens that are

445   sometimes responsible for unapparent and/or latent disease, thus replacing or

446   reducing the use of the invasive diagnostic methods currently widely used to

447   make diagnoses and to carry out health monitoring in fish populations.

448   However, the detection of disease-causing pathogens during chronic

449   infections has different limitations and is more difficult compared to detection

450   and identification during the acute infection phase.

451 A good level of health surveillance has been achieved, today, in our zebrafish

452 facility due to the application of a reliable health monitoring program,

453 characterized by daily checks of the fish health status, routine and non-routine

454 total body histopathological evaluation of sentinel, dead and diseased fish,

455 supported by ancillary assays, close observance of hygienic procedures and

456 egg bleaching of incoming fish. Respect of Standard Operative Procedures

457 (SOPs) and, in particular, attention to biosecurity, i.e. animal transfers

458 between quarantine and main rooms, and bleaching eggs, will avoid infection

459 with pathogens that cause clinical disease and that could directly change

460 results or increase the variability or a lack of reproducibility of experiments.

461 Based on our experience, outbreaks of infectious diseases, and also the latent

462 presence of infectious microorganisms, may be frequently recorded in fish

463 coming from aquaculture industries, ornamental fish trade and wild-caught

464 animals (obviously the latter cannot be provided with reports on health

465 status). They could affect experimental trial feasibility and the analysis of

466 experimental results increasing the variability of measured parameters among

467 experimental units. For these reasons, to partially overcome the above-

468 mentioned problems, in the case in which no purpose-bred fish are available,

469 farmed fish subjected to periodic health checks should be preferred rather

470 than wild-caught animals. For ornamental fish, eggs, fries and alevins

471 produced within the quarantine zone should be preferred rather than directly

472 introduce and use fish acquired from aquarium shops. Some considerations

473 can be made on the EU regulation, which, since its application, has promoted

474 a new perception of animal care and welfare. Almost all the diseases here

475 described were registered before the application of the new Italian law D. Leg.

476 26/2014, in application of the EU Directive 63/2010. Certainly, the new

477 regulations will impose higher attention in all phases, from production to

478 rearing and experimenting with laboratory fish, safeguarding the health of

479 animals and guaranteeing the quality of experiments and results; the quality

480 of experiments will be guaranteed through certified specific competences.

492 **Ethical considerations**

493 This article does not contain any studies involving human participants or

494 animals performed by any of the authors.

References

495   **References**

496   1.   Lipman NS, Perkins SE. Factors that may influence animal research.

497   In: *Laboratory Animal Medicine (Second Edition)*. Elsevier, 2002,

498   pp. 1143–1184.

499   2.   Baker DG. Natural pathogens of laboratory mice, rats, and rabbits

500   and their effects on research. *Clin Microbiol Rev* 1998; 11: 231–266.

501   3.   Ostrander GK. The Handbook of Experimental Animals: The

502   Laboratory Fish. 2000; 606.

503   4.   Dahm R, Geisler R. Learning from small fry: the zebrafish as a

504   genetic model organism for aquaculture fish species. *Mar Biotechnol*

505   2006; 8: 329–345.

506   5.   Kari G, Rodeck U, Dicker AP. Zebrafish: an emerging model system

507   for human disease and drug discovery. *Clin Pharmacol Ther* 2007;

508   82: 70–80.

509   6.   Mitchell D, Paniker L, Sanchez G, et al. The etiology of sunlight-

510   induced melanoma in Xiphophorus hybrid fish. *Mol Carcinog Publ*

511   *Coop with Univ Texas MD Anderson Cancer Cent* 2007; 46: 679–

512   684.

513   7.   Brittijn SA, Duivesteijn SJ, Belmamoune M, et al. Zebrafish

514   development and regeneration: new tools for biomedical research. *Int*

515   *J Dev Biol* 2009; 53: 835–850.

516   8.   MacRae CA, Peterson RT. Zebrafish as tools for drug discovery. *Nat*

517   *Rev Drug Discov* 2015; 14: 721.

518   9.   Lawrence C, Ennis DG, Harper C, et al. The challenges of

519   implementing pathogen control strategies for fishes used in

520   biomedical research. *Comp Biochem Physiol Part C Toxicol*

521   *Pharmacol* 2012; 155: 160–166.

522   10.   Matthews JL, Brown AM V, Larison K, et al. Pseudoloma

523   neurophilia ng, n. sp., a new microsporidium from the central nervous

524   system of the zebrafish (Danio rerio). *J Eukaryot Microbiol* 2001; 48:

525   227–233.

526   11.   Broussard GW, Norris MB, Schwindt AR, et al. Chronic

527   Mycobacterium marinum infection acts as a tumor promoter in

528   Japanese Medaka (Oryzias latipes). *Comp Biochem Physiol Part C*

529   *Toxicol Pharmacol* 2009; 149: 152–160.

530   12.   Collymore C, Crim MJ, Lieggi C. Recommendations for health

531   monitoring and reporting for zebrafish research facilities. *Zebrafish*

532   2016; 13: S-138.

533   13.   Kent ML, Harper C, Wolf JC. Documented and potential research

534   impacts of subclinical diseases in zebrafish. *ILAR J* 2012; 53: 126–

535   134.

536   14.   Martins S, Monteiro JF, Vito M, et al. Toward an integrated zebrafish

537   health management program supporting cancer and neuroscience

538   research. *Zebrafish* 2016; 13: S-47.

539   15.   Spagnoli ST, Xue L, Murray KN, et al. Pseudoloma neurophilia: A

540   retrospective and descriptive study of nervous system and muscle

541   infections, with new implications for pathogenesis and behavioral

542   phenotypes. *Zebrafish* 2015; 12: 189–201.

543   16.   Spagnoli S, Xue L, Kent ML. The common neural parasite

544   Pseudoloma neurophilia is associated with altered startle response

545   habituation in adult zebrafish (Danio rerio): Implications for the

546   zebrafish as a model organism. *Behav Brain Res* 2015; 291: 351–360.

547   17.   Hegedűs Z, Zakrzewska A, Ágoston VC, et al. Deep sequencing of

548   the zebrafish transcriptome response to mycobacterium infection.

549   *Mol Immunol* 2009; 46: 2918–2930.

550   18.   van der Sar AM, Spaink HP, Zakrzewska A, et al. Specificity of the

551   zebrafish host transcriptome response to acute and chronic

552   mycobacterial infection and the role of innate and adaptive immune

553   components. *Mol Immunol* 2009; 46: 2317–2332.

554   19.   Stead SM, Laird L. *The handbook of salmon farming*. Springer

555   Science & Business Media, 2002.

556   20.   Kent ML, Kieser D. Avoidance of introduction of exotic pathogens

557   with Atlantic salmon reared in British Columbia. *Biosecurity Aquac*

558   *Prod Syst Exclusion Pathog Other Undesirables* 2003; 43–50.

559 21. Barton CL, Johnson EW, Tanguay RL. Facility design and health

560   management program at the Sinnhuber Aquatic Research Laboratory.

561   *Zebrafish* 2016; 13: S-39.

562 22. Geisler R, Borel N, Ferg M, et al. Maintenance of zebrafish lines at

563   the European zebrafish resource center. *Zebrafish* 2016; 13: S-19.

564 23. Murray KN, Varga ZM, Kent ML. Biosecurity and health monitoring

565   at the Zebrafish International Resource Center. *Zebrafish* 2016; 13:

566   S-30.

567 24. Thrusfield M, Christley R. *Veterinary epidemiology*. Wiley Online

568   Library, 2005.

569 25. Harper C, Lawrence C. *The laboratory zebrafish*. Crc Press, 2016.

570 26. Coyle SD, Durborow RM, Tidwell JH. *Anesthetics in aquaculture*.

571   Southern Regional Aquaculture Center Stoneville, 2004.

572 27. Leary SL, Underwood W, Anthony R, et al. AVMA guidelines for

573   the euthanasia of animals: 2013 edition. American Veterinary

574   Medical Association Schaumburg, IL, 2013.

575 28. Marino F, Germanà A, Panebianco A. A case of schwannoma in

576   farmed seabream Sparus aurata. *Dis Aquat Organ* 2008; 82: 249–252.

577 29. Kobayasi H. Studies on Trematoda in Hainan Island. I. Determination

578   of the second intermediate hosts of trematodes and trematodes found

579 in the intestinal tract of dogs by experimental feeding with fish in

580 Hainan-Island. *Japanese J Med Sci V Pathol* 1942; 6: 181–185.

581 30. Marino F, Lanteri G, Rapisarda G, et al. Spontaneous schwannoma in

582 zebrafish, Danio rerio (Hamilton). *J Fish Dis* 2012; 35: 239–242.

583 31. Zon LI, Peterson RT. In vivo drug discovery in the zebrafish. *Nat Rev*

584 *Drug Discov* 2005; 4: 35.

585 32. McCollum CW, Ducharme NA, Bondesson M, et al. Developmental

586 toxicity screening in zebrafish. *Birth Defects Res Part C Embryo*

587 *Today Rev* 2011; 93: 67–114.

588 33. Zoupa M, Machera K. Zebrafish as an Alternative Vertebrate Model

589 for Investigating Developmental Toxicity—The Triadimefon

590 Example. *Int J Mol Sci* 2017; 18: 817.

591 34. McGrath P, Li C-Q. Zebrafish: a predictive model for assessing drug-

592 induced toxicity. *Drug Discov Today* 2008; 13: 394–401.

593 35. Selderslaghs IWT, Van Rompay AR, De Coen W, et al. Development

594 of a screening assay to identify teratogenic and embryotoxic

595 chemicals using the zebrafish embryo. *Reprod Toxicol* 2009; 28:

596 308–320.

597 36. Brannen KC, Panzica-Kelly JM, Danberry TL, et al. Development of

598 a zebrafish embryo teratogenicity assay and quantitative prediction

599 model. *Birth Defects Res Part B Dev Reprod Toxicol* 2010; 89: 66–

600   77.

601   37.   Hermsen SAB, van den Brandhof E-J, van der Ven LTM, et al.

602   Relative embryotoxicity of two classes of chemicals in a modified

603   zebrafish embryotoxicity test and comparison with their in vivo

604   potencies. *Toxicol Vitr* 2011; 25: 745–753.

605   38.   Loucks E, Carvan III MJ. Strain-dependent effects of developmental

606   ethanol exposure in zebrafish. *Neurotoxicol Teratol* 2004; 26: 745–

607   755.

608   39.   Astrofsky KM, Schrenzel MD, Bullis RA, et al. Diagnosis and

609   management of atypical Mycobacterium spp. infections in established

610   laboratory zebrafish (Brachydanio rerio) facilities. *Comp Med* 2000;

611   50: 666–672.

612   40.   Prouty MG, Correa NE, Barker LP, et al. Zebrafish-Mycobacterium

613   marinum model for mycobacterial pathogenesis. *FEMS Microbiol*

614   *Lett* 2003; 225: 177–182.

615   41.   Kent ML, Whipps CM, Matthews JL, et al. Mycobacteriosis in

616   zebrafish (Danio rerio) research facilities. *Comp Biochem Physiol*

617   *Part C Toxicol Pharmacol* 2004; 138: 383–390.

618   42.   Watral V, Kent ML. Pathogenesis of Mycobacterium spp. in

619   zebrafish (Danio rerio) from research facilities. *Comp Biochem*

620   *Physiol Part C Toxicol Pharmacol* 2007; 145: 55–60.

621   43.   Whipps CM, Dougan ST, Kent ML. Mycobacterium haemophilum

622   infections of zebrafish (Danio rerio) in research facilities. *FEMS*

623   *Microbiol Lett* 2007; 270: 21–26.

624   44.   Whipps CM, Lieggi C, Wagner R. Mycobacteriosis in zebrafish

625   colonies. *ILAR J* 2012; 53: 95–105.

626   45.   Sanders JL, Watral V, Kent ML. Microsporidiosis in zebrafish

627   research facilities. *ILAR J* 2012; 53: 106–113.

628   46.   Doménech A, Fernández-Garayzábal J, Garcia J, et al. *Association of*

629   *Pseudomonas anguilliseptica infection with 'winter disease' in sea*

630   *bream, Sparus aurata L*. 2001. Epub ahead of print 24 December

631   2001. DOI: 10.1046/j.1365-2761.1999.00124.x.

632   47.   Muniesa A, Ruiz-Zarzuela I, de Blas I. Design and implementation of

633   a collaborative epidemiological surveillance system for aquaculture

634   (VECA). *Rev Aquac* 2018; 10: 370–375.

635   48.   Yanong RPE. Lymphocystis disease in fish. *Ext Publ FA181 Univ*

636   *Florida, Gainsv*.

637   49.   Tsertou MI, Smyrli M, Kokkari C, et al. The aetiology of systemic

638   granulomatosis in meagre (Argyrosomus regius): The "Nocardia"

639   hypothesis. *Aquac Reports* 2018; 12: 5–11.

640   50.   Francis-Floyd R. *Mycobacterial infections of fish*. Southern Regional

641   Aquaculture Center, 2011.

642 51. Johansen R, Needham JR, Colquhoun DJ, et al. Guidelines for health

643   and welfare monitoring of fish used in research. *Lab Anim* 2006; 40:

644   323–340.

645

646

647

648

649

650

651

652

653

654

655

656

657

658

659

660

661 **Tabel 1**. Spontaneous diseases registered for each fish species during 10

662   years of activity of the facility and their impact level on experimental

663   research.

Specie	Used Fish	Diseased Fish	Pathologies	Effects on research
Danio rerio	1465	24	Congenital abnormalities	exclusion
		8	*Mycobacterium*	severe
		1	Schwannoma	none
		4	cartilaginous cysts in the gills	low
Mugil cephalus	340	4	heterophidosis	low
		60	mycobacteriosis	severe
Sparus aurata	323	32	lymphocystic disease	medium/severe
		25	*Cryptocarion irritans*	mortality
		23	*Enteromyxum leei*	low
		1	Winter Disease	mortality
		70	*Ceratomyxa* sp.	low
Dicentrarchus labrax	296	8	vibriosis	severe
		25	pasteurellosis	severe
		2	*Amyloodinium ocellatum*	medium
		58	Sphaerosporosis	low
Argyrosomus regius	80	40	Non-infectious systemic granuloma	none
		6	Cryptocarioniasis	mortality
Dentex dentex	80	0	none	
Cyprinus carpio	60	0	none	
Boops boops	20	0	none	
Tinca tinca	20	0	none	
Anguilla anguilla	20	0	none	
Carassius auratus	16	4	*Myxobolus*	severe
		3	schwannoma	none
		5	mycobacteriosis	severe
		1	polycystic kidney	medium
		15	ammonia intoxication	severe
Poecilia reticulata	70	14	mycobacteriosis	severe
Xiphophorus variatus	30	2	mycobacteriosis	severe
Total Number	**2820**	**411**		

664

665 None: diseases that do not have particular effects on research procedures; low: diseases with a low

666 impact on research procedures, that do not change results of the study; severe: diseases with a high

667 impact on research, this pathology can invalidate the analysis and the study; mortality: diseases causing

668 mortality and consequently invalidate the study; exclusion: diseases that need the exclusion of fish from

669 research, because they could invalidate the study.

670   **Figure Legends**:

671   **Figure 1**. *Centrocestus* sp. larvae within cartilaginous cyst in zebrafish gills

672   (H&E 20x).

673   **Figure 2**. Cytological imprinting smear obtained from nodules showing

674   *Myxobolus* sp. in goldfish (May Grunwald Giemsa 40x).

675   **Figure 3.** Macroscopical feature of schwannoma in goldfish fin.

676   **Figure 4**. Photobacteriosis in sea bass. Note enlarged spleen with

677   necrotic/granulomatous foci.

678   **Figure 5**. Detail showing hypertrophic dermal fibroblast with viral

679   cytoplasmic inclusions (H&E 40x). Arrows: cytoplasmic viral inclusion.
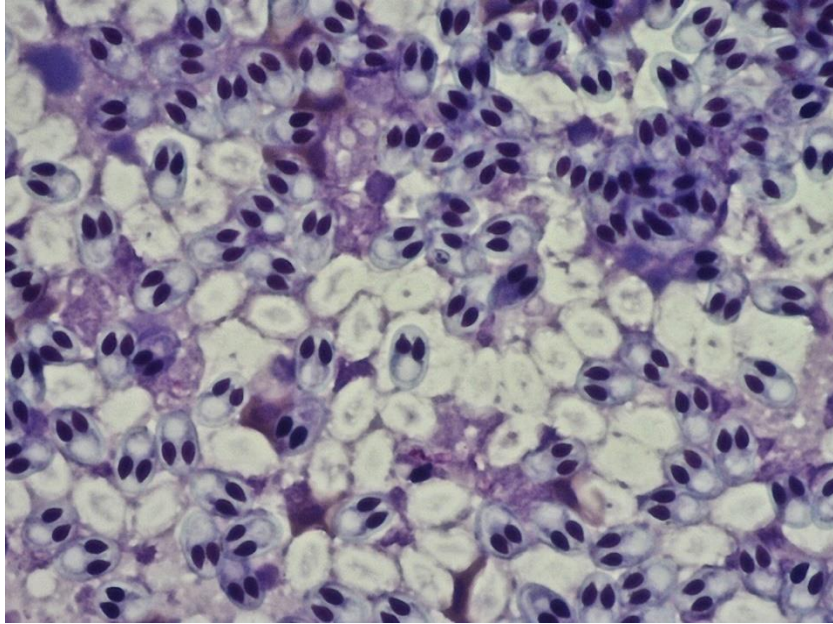
680

681   **Figure 1**
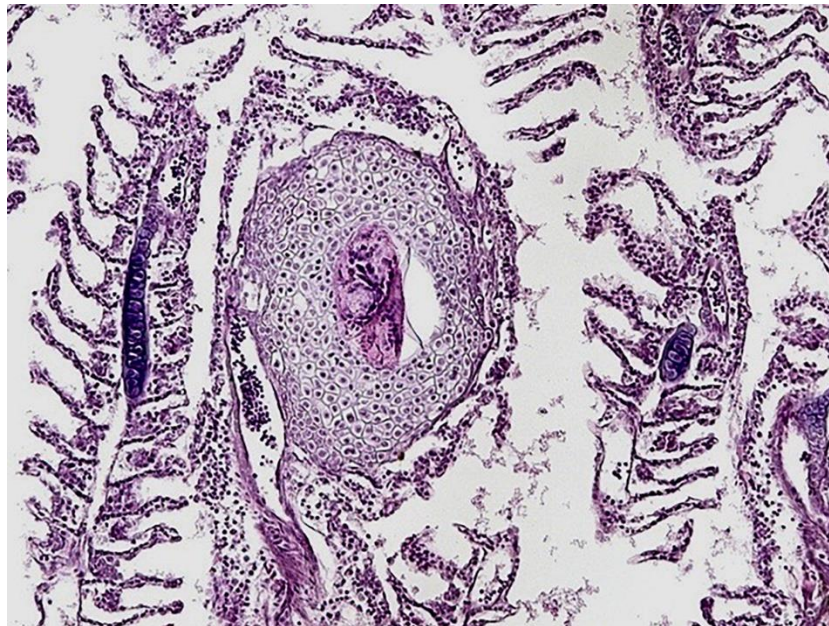
682

683

684   **Figure 2**

685

686

687   **Figure 3**

688

689

690   **Figure 4**

691

692

693   **Figure 5**

694