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Occurrence of diseases in fish used for experimental research

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

2

3

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20

21 **Abstract**

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

43 **Introduction**

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

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

52 In the last few years, a field that is arousing growing interest is the use of fish
53 as experimental animals for biomedical research, both for human research and
54 for aquaculture purposes. However, little is known on the pathogens of fish
55 compared to mice, rats and rabbits; information regarding pathological
56 surveys or the health status of fish used in experimental trials is scarce. This
57 is probably due to the fact that, despite an increasing use of a wide range of
58 species of fish in several research projects, no particular attention has been
59 paid to health management in the field of laboratory animals: with regard to
60 the use of fish there is not even a suitable pathogen control at fish facilities.^{3–}

61 ⁸ This aspect, which has unfortunately been neglected, could be a useful tool
62 to control the biological and experimental variability of fish used in research
63 procedures, providing useful information for a correct interpretation of
64 results.⁹ However, given that pathogens can affect fish in a very similar

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 data 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

96 **Materials and Methods**

97 *The facility*

98 This study was carried out at the University of Messina, CISS establishment.
99 The laboratory has been recognized by the Italian Ministry of Health since
100 2006 and was authorized as an establishment for the production of fish for
101 experimental purposes in 2010. The CISS facility has 3 rooms: a separate
102 quarantine room, a zebrafish breeding room and a main room, in which all
103 experiments are carried out. The main room is equipped with 50 fiberglass
104 and plastic tanks with a capacity of 120 to 800 liters, each one provided with
105 independent biological-sand and activated carbon filters, aerator pump and
106 automated monitoring system for continuous water parameter controls
107 (Oxywifi2, Tecnos, Chioggia, Italy).
108 Zebrafish maintained in the zebrafish breeding room are reared, in a ZebTEC
109 Active Blue Stand Alone system, Tecniplast. In these three recirculating
110 housing systems, the water is sterilized with UV (minimum dose 135,000
111 $\mu\text{Wsec}/\text{cm}^2$) and derives from reverse osmosis-treated city water to which is
112 added salt to a conductivity of 450 μS . Environmental conditions at the
113 primary enclosure (water tanks) are maintained at 27 ± 1 °C temperature
114 values, pH 7.2 ± 0.3 , dissolved oxygen content (DO) of 6.00 ppm for
115 freshwater species. Marine species are held in seawater, artificially
116 reconstituted by the addition of salt (Blue Treasure Sea Salt, Qingdao Sea-
117 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*

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).

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

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

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

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 $\mu\text{g ml}^{-1}$) 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 Genbank, by WU BLAST 2 software.

222 *Lymphocystivirus* 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 (craniosynchosis 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 *Centrocestus*
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 damsela* 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

343 **Discussion**

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

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

351 In the last few years, the use of zebrafish has increased considerably in many
352 fields of research. Low breeding costs, high prolificity of the broodstock,
353 rapid organogenesis, transparency of embryos and the extensively studied
354 development process have all made the zebrafish a valuable animal model to
355 study organogenesis disorders, test novel therapies and screen toxicity of
356 chemicals and potential teratogens.³¹ In particular, zebrafish embryos provide
357 a reliable model for the high-throughput screenings of chemicals as they
358 present the advantages of an in vivo model and the convenience of an in vitro
359 approach.^{32,33} Recently, the use of zebrafish for development toxicology
360 assays has been encouraged given the high concordance with animal data that
361 has been demonstrated.^{34–37} Zebrafish developmental toxicity assays
362 (ZEDTA) have a good predictive value regarding assessing chemicals or
363 candidate drugs for human safety. However, the use of various zebrafish lines
364 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

our facility, histological and molecular examinations did not show any evidence of this pathogen in either the cut brain or spinal cord tissue.⁴⁵ European sea bass coming from fish farms, can develop vibriosis and photobacteriosis. These two infectious diseases may interfere on experiment feasibility causing a high rate of mortality (90-100% in acute forms). Sphaerosporosis is a parasitic disease due to the myxozoa *S. dicentrarchi*. These protozoa can be endemic in some fish farms; it is an opportunistic pathogen, generally with low pathogenicity that can sometimes cause severe tissue changes and death. In our study, 58 *D. labrax* out of a total of 296 showed few myxosporeans found in the *muscularis mucosae* of the intestine without any signs of inflammation.

Regarding the dinoflagellate *A. ocellatum* that we found in two specimens of *D. labrax*, the low parasitic charge, low incidence of disease and low tissue damage observed in the gills did not interfere with results of the trial.

The protozoans *C. irritans*, *E. leei* and *Ceratomyxa* sp. that were found in some *S. aurata* fish during our study are frequently detectable in this species. *C. irritans* under experimental conditions can be lethal, with a very high percentage of mortality and thus must be immediately identified and excluded from experimental trials. *E. leei* and *Ceratomyxa* sp usually show low pathogenicity for gilthead sea bream.

‘Winter disease’ is a metabolic syndrome common in captive sea bream 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 system⁴⁸.
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-hypovitaminosis⁴⁹.
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.

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492 **Ethical considerations**

493 This article does not contain any studies involving human participants or
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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
<i>Danio rerio</i>	1465	24	Congenital abnormalities	exclusion
		8	<i>Mycobacterium</i>	severe
		1	Schwannoma	none
		4	cartilaginous cysts in the gills	low
<i>Mugil cephalus</i>	340	4	heterophidiosis	low
		60	mycobacteriosis	severe
<i>Sparus aurata</i>	323	32	lymphocystic disease	medium/severe
		25	<i>Cryptocaryon irritans</i>	mortality
		23	<i>Enteromyxum leei</i>	low
		1	Winter Disease	mortality
		70	<i>Ceratomyxa</i> sp.	low
<i>Dicentrarchus labrax</i>	296	8	vibriosis	severe
		25	pasteurellosis	severe
		2	<i>Amyloodinium ocellatum</i>	medium
		58	Sphaerosporosis	low
<i>Argyrosomus regius</i>	80	40	Non-infectious systemic granuloma	none
		6	Cryptocaryoniasis	mortality
<i>Dentex dentex</i>	80	0	none	
<i>Cyprinus carpio</i>	60	0	none	
<i>Boops boops</i>	20	0	none	
<i>Tinca tinca</i>	20	0	none	
<i>Anguilla anguilla</i>	20	0	none	
<i>Carassius auratus</i>	16	4	<i>Myxobolus</i>	severe
		3	schwannoma	none
		5	mycobacteriosis	severe
		1	polycystic kidney	medium
		15	ammonia intoxication	severe
<i>Poecilia reticulata</i>	70	14	mycobacteriosis	severe
<i>Xiphophorus variatus</i>	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).

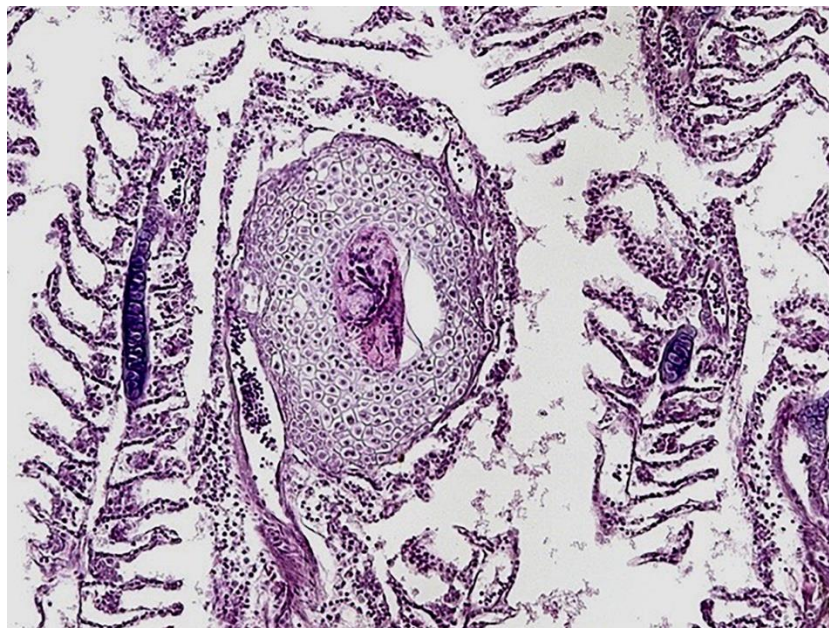
675 **Figure 3.** Macroscopical feature of schwannoma in goldfish fin.

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.

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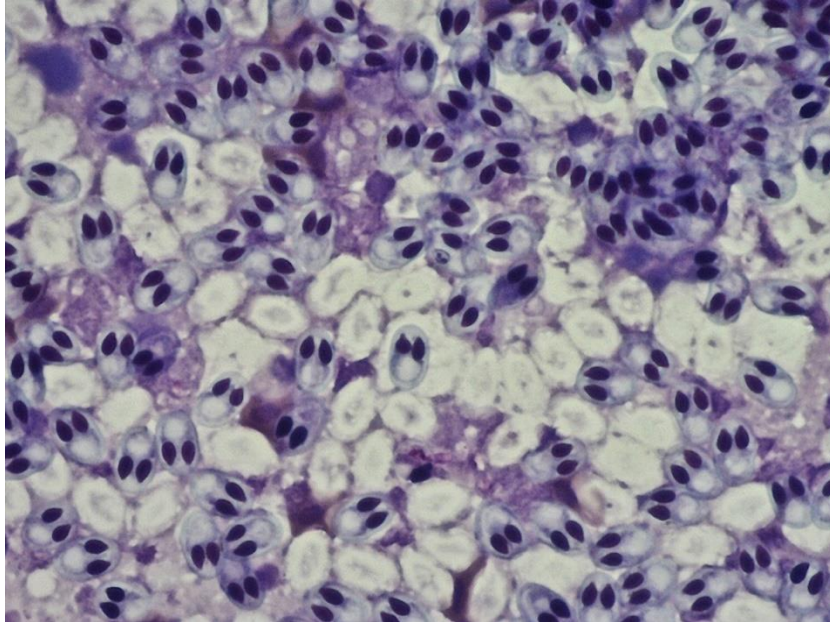
681 **Figure 1**



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684 **Figure 2**



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687 **Figure 3**



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690 **Figure 4**



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693 **Figure 5**



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