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RESAMA: A Network for Monitoring Health and Husbandry Practices in Aquatic Research Facilities

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Abstract

Health monitoring is a crucial aspect of the management of any research animal house. RESAMA is a network strong of 60 academic and private partners acting in France since the end of 2012. The network aims to increase awareness of animal caretakers and researchers on health management issues in facilities holding aquatic model species (zebrafish, *Xenopus*, medaka, Mexican tetra). To do so, each partner research facility will be visited at least once. The visiting team is composed at least of one veterinarian and one zootechnician specialized in aquatic species. The visit results in a health-monitoring assessment of the facility, which includes a sampling for histo-pathological, bacteriological, and molecular pathogen detection. During the visit, rearing practices are also reviewed through an interview of animal caretakers. However, the present report essentially focuses on the health-monitoring aspect. The ultimate goal of the project is to provide a network-wide picture of health issues in aquatic facilities. Performed in parallel, the rearing practice assessment will ultimately help to establish rational relationship between handling practices and animal health in aquatic facilities. The study is still in progress. Here, we describe the results to be drawn from an analysis of the 23 facilities that had been visited so far. We sampled 720 fish and 127 amphibians and performed a little less than 1400 individual tests.

Introduction

Over the past decades, fishes and amphibians have gained a new role as model organisms in human biology and biomedical studies.[1] However, advances in husbandry and health management for these species have lagged well behind developments in science and technological innovation. Moreover, an increasing number of studies evaluate fishes or frogs for weeks or months, so defining the health status of the colony becomes critical to prevent artifact associated with underlying infections.[2,3] Nevertheless, the landscape is bare compared to the wealth of health data available for mammalian models: knowledge is sparse and relatively disorganized. Health of aquatic animals remains in most facilities mainly unmanaged, and few veterinarians are trained to deal with aquatic model species. As a consequence, sea salt and malachite green remain the ultimate health management tools for many caretakers. Meanwhile, there is a growing awareness that this trend must change.

This background led to the creation of a French health-monitoring network composed of research aquatic facilities. The acronym of the network is RESAMA (Réseau d'Études Sanitaires des Animaux Modèles Aquatiques: Network for the Study of Aquatic Model Animal Health). The first aim of the network is to increase the knowledge on pathogen and health issues in amphibian and fish facilities, the second is to improve husbandry and health management practices. RESAMA is now a network strong of 60 partners located mainly in France (and 1 UK facility at the time of writing). Each partner facility will be visited at least once during the time course of the project, which is financed until 2019. The network is still recruiting partners.

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The RESAMA is mainly focused on the aquatic species that are most commonly used in research: *Danio rerio* Buchanan-Hamilton 1822 (zebrafish) and *Xenopus laevis* Daudin 1802 (African clawed frog). Indeed, other amphibians or fish species less frequently encountered are also incorporated in the study: we present here some data for *Xenopus tropicalis* Gray 1864 (Western clawed frog), *Oryzias latipes* Temminck and Schlegel 1846 (medaka rice fish), or *Astyanax mexicanus* De Filippi 1854 (Mexican tetra).

Although veterinarian data about aquatic model animals are not as thorough as those accumulated on mammalian species, still quite a sizable corpus of data is already available. *Xenopus* are kept in research laboratories since the beginning of the 20th century and a significant corpus of health-related observations exists.[3,4] For example, mycobacteriosis is not an unusual problem among poikilothermic vertebrates and their association with captive aquatic species has long been noted.[5–9] Multiple species of *Mycobacterium* spp. have been isolated from captive amphibian or fish species, including *Mycobacterium marinum*, *Mycobacterium fortuitum, Mycobacterium chelonae*, *Mycobacterium gordonae*, *Mycobacterium liflandii,* and *Mycobacterium szulgai*[6–12] to cite a few. Most of these are potential zoonosis and can infect a wide spectrum of vertebrate species.

Microsporidia are well recognized pathogens of fishes. A microsporidian infecting the central nervous system of zebrafish was first reported in 1980 by a group in France, in fish purchased from a pet store for use in toxicological studies.[13] The parasite has thereafter been found infecting zebrafish in many research facilities, and is now known as *Pseudoloma neurophilia*.[14] The parasite affects the central nervous system, muscles, and ovaries.[15] Tell-tale signs of infection are severe emaciation and lordosis. Yet, such signs are not pathognomic as many things can produce similar lesions, including other infectious diseases (such as mycobacteriosis), fish manipulation, or simply aging. Conversely, during early infection phases, the infected fish are virtually indistinguishable from their noninfected siblings.[16] *P. neurophilia* is the most commonly observed microsporidian parasite of zebrafish and may be the most widespread pathogen in zebrafish facilities. The infection was detected in more than 74% of the facilities examined through the Zebrafish International Resource Center diagnostic service in 2010.[17]

In this article, we present the first summary to be drawn from an analysis of 23 aquatic facilities visited since 2013. Each facility was visited at least once and audited for both health status and husbandry practices.

Materials and Methods

Definition of an independent research facility

We settled for a definition easy to go by: two facilities are independent if the majority of the facility management work load is done by persons who are different. Although this may seem obvious, the question arose for campus-wide centralized animal houses. Frequently, each research team livestock is physically separated from the others (using dedicated tank racks or rooms); frequently as well some equipment, consumables, or personnel are shared (water production, feeding, etc.). Then, to decide whether the two facilities are independent or not, one has to refer to the presence of fish care staff dedicated to a team and to the implication of nonspecialist staff (laboratory technician and researchers, PhD students or undergraduates) in the day-to-day management of the facility.

Protocol of the sanitary visit

The visits were organized as follows: at least one veterinarian and one specialized animal caretaker composed the team that carried out the visit, which took the form of a classical sanitary visit. To start, a brief presentation of the aims of the network was made to the staff. Then, the veterinarian visit began with a historical review of the facility (results of previous health monitoring, existence of previous epizooty, mortality rate, pharmacy, etc.) followed by the health monitoring and sampling visit of the facility. Meanwhile, a zootechnic assessment was organized coupling staff interviews and a tour of the facility. This allowed to review the design of the facility (technical solutions chosen, water production system, day/night cycle, etc.) as well as Standard Operation Protocols (water parameter controls, feeding practices, strain management, etc.).

Sampling procedure

Animals were sampled from each water unit defined as all the tanks belonging to the same water recirculating loop. The veterinarian selected the animals to be sampled. Depending on the global sanitary state of the facility, the animals were sampled as follows: (1) If symptomatic, clinical sign-bearing animals were present in the water unit: those animals were selected first. If several animals carried similar clinical signs, the most affected animal was selected in priority. If several animals bore different sets of clinical signs, possibly indicative of different diseases, each set was sampled. (2) If no (more) symptomatic animals was/were found in the water unit: one or several randomly selected animals were chosen; if sentinel animals were present, those were chosen in priority. After selection, the animals were fished out from the tank by the staff of the facility by using a net and transferred into individual recipient-containing water. They were then transported to the dissecting room.

Necropsy

All animals were euthanized using an overdose of benzocaine (250 mg/L) in accordance with European Community (EC) recommendations for euthanasia of laboratory animals.[18] An external examination was carried out to detect any lesion. For fish, about half of the samples were necropsied, the rest were kept for histological analysis. Necropsy was conducted following Noga's postmortem techniques.[19] Cutaneous mucus, branchial arches, pectoral and caudal fins, and any lesion were systematically observed under a microscope. Each internal organ was subjected to a macroscopic observation and observed between cover glass and microscope slide. The digestive tract and its content were subjected to a thorough observation to find parasites or helminth eggs. For amphibians, all the animals went through necropsy. Skin scrap of the belly and the back was performed and each organ was subjected to a macroscopic observation. Skin, lung, gall bladder, urinary bladder, gut (first, middle, and posterior parts), and its content were carefully examined under a microscope.

Histology

Tissues and organs fixed in 10% buffered formalin (Q Path, VWR) were then processed by standard paraffin wax techniques. Before embedding, fishes were cut apart in parasagittal length and both parts were held in the same mold. Samples were sectioned with microtome at 4 μm thickness and stained with hematoxylin, eosin, and saffron, and Fite-Faraco. Dedicated specific stains (Giemsa, Gram, PAS) were performed as necessary.

Bacteriology

When a bacterial infection was suspected, bacteriological analyses were carried out. A sample of blood was taken by cardiac puncture. For fishes, a posterior kidney biopsy was taken by a dorsal approach. For amphibians, spleen, liver, and kidney were sampled by a ventral approach. The remaining of the tested individual was then fixed for further histological analysis. The samples were sent within 24 h to the laboratory for isolation, identification, and antimicrobial disk susceptibility tests. Samples were streaked on Anacker–Ordal and Tryptic Soy agar and incubated at 20°C ± 2°C for 14 days. Bacterial colonies were identified using the Maldi-Tof (matrix-assisted laser desorption/ionization time-of-flight analyzer) technique or API® identification strips (Biomérieux) and kept frozen at −80°C. Antimicrobial disk susceptibility tests were performed according to Clinical & Laboratory Standards Institute (CLSI) guidelines.[20]

Polymerase chain reaction analysis

Molecular analysis through polymerase chain reaction (PCR) was initiated on internal organs to search for several infectious diseases. The analysis was performed on biopsies taken from a single specimen. A subset of PCR assays was performed on internal organs such as spleen, liver, heart, gonads, and kidneys (excluding gut) that were pooled from several animals (Supplementary Tables S1 and S2; Supplementary Data are available online at www.liebertpub.com/zeb). For *Batrachochytrium dendrobatidis* and *Batrachochytrium salamandrivorans*, a skin swab of the entire belly and legs of *Xenopus* was performed. Samples were immediately frozen and sent under negative temperature using a dedicated transporter (TSE express medical) to the laboratory for analysis. The DNA was extracted following the protocol described by Hyatt *et al.*[21] and stored at −20°C. The range of pathogens tested was different depending on the species considered. We tested the presence of *Mycobacterium* spp. in several specimens from each species. PCR for *Mycobacterium* sp. was performed by real-time PCR targeting *hsp65* sequence using primers from Telenti *et al.*[22] (5′-ACCAACGATGGTGTGT CCAT and 5′-CTTGTCGAACCGCATACCCT), which are present in all *Mycobacterium* species; identification was attempted using the GenoType AS/CM assay kit (Hain Lifescience GmbH) and then confirmed by the sequencing of *hsp65* gene. In zebrafish, to detect *P. neurophilia*, real-time PCR was carried out on spine and brain following the protocol and using primers described by Sanders and Kent.[23] Real-time PCR for *Batrachochytrium* was performed according to Boyle *et al.*[24] for *B. dendrobatidis*, Martel *et al.*[25] for *B. salamandrivorans*, using the primers and PCR conditions described in those articles. The PCR amplifications for *Ranavirus* were performed using PCR Master Mix (Promega). A first PCR run was performed with primers 5′nMCP (5′-GCAGCAGTTT TCGGTCGGCG) and 3′nMCP (5′-CGCTTGGCCTCTGGC ATGGT) according to Mao *et al.*[26]

Implementation of a health-monitoring oriented database and its web front end

To ease the treatment of the data, we set a database to specifically hold sanitary visit results. In brief, the database allows persons in charge of the network to access the full extent of the bacteriological and histopathological results. Each facility manager disposes of a direct access to the results concerning his/her facility but has no access to the results of the other facilities. The database is based on a drupal content management system (version 7, www.drupal.org) and 40 or so modules. The source code and a short installation procedure can be found here: http://distro.resama.aquario.fr, alongside some screenshot of the application.

Results

Health analysis of the amphibian facilities

Seven *X. laevis* facilities housing from 150 to 4500 animals were visited (Table 1). Four facilities also held *X. tropicalis* (200–2000 animals). During the visits, 87 *X. laevis* were sampled, from which 60 were chosen because they displayed obvious sign of pathology (ascites, scoliosis, oedema, etc.). The 27 remaining were randomly chosen (Table 2). Thirty-nine *X. tropicalis* were also sampled (Tables 1 and 2). In total, 344 analyses were carried out, including autopsies (110),

Table 1. Sample Size and Number of Visited Facilities

				Sex ratio		
Species	*Visited facilities*	*Sampled animals*	*Analysis*	*Male*	*Female*	*ND*
Danio rerio	17	612	931	284	250	78
Oryzias latipes	4	85	115	46	29	10
Astyanax mexicanus	1	23	32	4	14	5
Xenopus tropicalis	4	39	75	13	24	2
Xenopus laevis	7	87	218	20	50	17
Xenopus borealis	1	1	3	1	0	0

Twenty-three facilities were visited: three housed several fish species, three several xenopus species, and three were holding both fish and amphibians. The number of animals sampled is roughly proportional to total population size. Sex ratio is globally respected except for *X. laevis* where more females were analyzed.

Table 2. Type of the Animals Analyzed

	Random sampling (asymptomatic)		Sentinelles		Targeted sampling (symptomatic)	
Species	*Sampled*	*Positives (%)*	*Sampled*	*Positives (%)*	*Sampled*	*Positives (%)*
D. rerio	104	3 (3)	36	6 (17)	472	176 (37)
O. latipes	25	1 (4)	3	0 (0)	57	21 (37)
A. mexicanus	14	4 (29)			9	8 (89)
X. tropicalis	9	2 (22)			30	12 (40)
X. laevis	27	12 (44)			60	43 (72)
X. borealis	1	0 (0)			0	0 (0)

Most of the animals sampled were chosen from symptomatic animals. Few facilities maintained sentinels and those who did only maintained pre-UV sterilization sentinels.

bacteriological assays (33), histological analyses (62), and PCR (67 against chytrid, 43 against mycobacteria, and 29 for *Ranavirus*). Choice of the analysis to be performed was dictated by the results of the initial gross examination or autopsy of the animal. In total, 55 animals were found to be infected (Tables 4 and 5). The most frequent infections were parasitic in nature. Protozoa were found in 35 occurrences (Table 4). Most of them could be described as commensal parasites such as *Balantidium xenopodis*, *Protoopalina xenopodus*, and *Trichodina xenopodus*.[27–29] *Pseudocapillaroides xenopi*, a skin nematode, was found in a single facility and was associated with sporadic high-mortality episodes as described by other authors.[30,31] *B. dendrobatidis* has been detected in three out of the seven facilities visited in 16 *X. laevis* and 2 *X. tropicalis*. Contrastingly, *Ranavirus* that has been linked to amphibian decline was not detected in the frogs sampled so far (data not shown). The most frequent bacterial agent found in *Xenopus* was *Aeromonas hydrophila*, one of the causative agents (among other bacteria) of the red-leg disease. *Mycobacterium* infection was a nonproblem in all but two of the frogs tested (Table 5). Some noninfectious diseases were recorded, gall bladder sludge in most cases. Sporadic nephrocalcinosis was significantly prevalent in one facility, and one case of Ovarian Hyperstimulation Syndrome was detected.[32]

Health analysis of the fish facilities

D. rerio number per facility ranged from 300 to 28,000 individuals. For *O. latipes*, the range was 150–12,000 animals. *P. neurophilia* and *Mycobacterium* spp. were the most common pathogens affecting zebrafish. Out of 247 zebrafish histologies, mycobacteria were detected in 46 specimens and *P. neurophilia* in 58. Coinfections were not rare (14 cases, all in symptomatic animals). *P. neurophilia* was detected at least in 116 animals out of the 410 animals tested (histology and/or PCR) in 14 facilities (Table 5). In two of those facilities, the presence of *P. neurophilia* seemed restricted to the quarantine area. Xenomas in brain were rare and limited to young animals. Older specimens showed more disseminated infection in the abdominal organs, mainly gonads, in muscles, and in kidneys. *Mycobacterium* infections come second with at least 84 cases out of 356 zebrafish analyzed. Again, 14 facilities were affected, in one of which the infection appeared limited to the quarantine area (Table 4). Species wise, *M. chelonae* was the most prevalent (10 facilities), followed by *M. gordonae* (5 facilities). It is worth noting that *M. gordonae* was also detected in all other tropical aquatic species (*X. tropicalis*, *O. latipes*, and *A. mexicanus*). Finally, *M. marinum* and *M. fortuitum*, the most virulent and zoonotic species, were rare, *M. marinum* was detected in one facility and *M. fortuitum* in two facilities. Both were limited to zebrafish colonies.

In zebrafish, no other parasite than *Pseudoloma* has been detected so far except *Flamingolepis liguloides*, which was detected in one facility in the gut of zebrafish fed with adult *Artemia salina* caught in the wild (Table 5). No parasites were observed in medaka fish facilities during necropsy and histology. The only fish parasite detected so far was an *Urecloides* spp., a specific gills monogenean parasite of the

Table 3. Summary of Zootechnical Parameters Used

		Type of water				Temperature (°C)		
Species	*Visited facilities*	*Mix tap water osmosed water*	*Osmosed water+salt*	*Minimal conductivity*	*Maximal conductivity*	*Min.*	*Max.*	*Median value*
D. rerio	17	9	8	150	800	24.4	28.5	27.4
O. latipes	4	4	0	150	200	26	28	27.8
A. mexicanus	1	1	0	550	NA	22[a]	27[a]	NA
X. laevis	7	6	1	450	1500	18	23	19.5
X. tropicalis	4	3	1	500	3000	24.1	26	25
X. borealis	1	1	0	550	NA	18	NA	18

Half of the zebrafish facilities used a mixture of filtered tap water and reverse osmosis water to reach the desired water hardness, the other half used osmosis water and artificial sea salt. One facility used freshwater ''discus'' salt. Temperature range is presented as minimal and maximal observed in Celsius. The median temperature is presented for each species.

[a]Cave populations of Astyanax are maintained at 22°C; surface populations at 27°C.

Table 4. Bacterial Species Repartition by Species

	D. rerio		*O. latipes*		*A. mexicanus*		*X. laevis*		*X. tropicalis*	
Bacteria detected	*Facilities*	*Animals*	*Facilities*	*Animals*	*Facilities*	*Animals*	*Facilities*	*Animals*	*Facilities*	*Animals*
Acinetobacter haemolitycus			1	2						
Acinetobacter junii	1	1								
Aeromonas caviae	4	4	1	4					1	1
Aeromonas hydrophila	4	4	1	2			5	7	1	2
Aeromonas salmonicida										
Aeromonas veronii	7	16	2	3						
A. veronii biovar sobria	6	13	2	3						
Chryseobacterium indologenes	1	1	1	1					3	1
Citrobacter freundii	2	3					2	2		
Mycobacterium (partial histology only)	14	43	3	5	1	7			1	1
Mycobacterium chelonae	11	23								
Mycobacterium fortuitum	2	4								
Mycobacterium gordonae	5	11	1	2					1	1
Mycobacterium marinum	1	1								
Mycobacterium mucogenicum	1	2								
Pseudomonas aeruginosa	1	1	1	1						
Pseudomonas fluorescens			1	1						
Pseudomonas putida	1	1	1	1					1	1
Shewanella (partial characterization only)	2	3								
Shewanella putrefaciens	2	2	1	1						
Stenotrophomonas maltophilia	2	2								
Vibrio (partial characterization only)	2	3								
Vibrio alginolyticus	1	2	1	1						

The table summarizes all the bacterial species found during the study. Only detected bacterial species are reported. spp. for some genus, the characterization was not always carried out until the exact species determination. For each fish or amphibian species, the table provides the number of facilities affected on the left and the number of infected animals on the right. Bacteria show little species specificity as far as the host is concerned.

A. mexicanus. Isolated bacteria belonged mostly to the motile *Aeromonas* group (*Aeromonas veronii*, *A. hydrophila*). None of those were associated with an excess of mortality rate in the animal house.

Histological analysis allowed us to assess infectious diseases and to identify numerous noninfectious diseases in zebrafish, such as nephrocalcinosis, neoplasia, egg-binding, and gut distension. Egg-binding, seminoma, and steatosis were very common in all the zebrafish facilities we sampled. Forty-five percent of the female displaying egg-binding also had granulomatous infections associated with acid-fast bacteria (most probably mycobacteriosis). We found 49 animals displaying steatosis. Such fish were found in most facilities (in 14 out of the 17 animal houses holding *D. rerio*). Affected animals were of age ranging from 2 months to 31 months. We found no evidence of sex bias (22 females, 25 males, and

Table 5. Distribution of Parasitic and Fungus Species

	D. rerio		*O. latipes*		*A. mexicanus*		*X. laevis*		*X. tropicalis*	
Bacteria detected	*Facilities*	*Animals*	*Facilities*	*Animals*	*Facilities*	*Animals*	*Facilities*	*Animals*	*Facilities*	*Animals*
Flamingolepis liguloides	1	4								
Batrachochytrium dendrobatidis							3	16	2	2
Saprolegnia sp.					1	2				
Pseudoloma neurophilia	14	116								
Probably *P. neurophilia*	3	4								
Capillaria xenopodis							1	5		
Balantidium sp.							6	24		
Protoopalina sp.							5	21		
Spironucleus sp.							4	12	1	1
Vorticella sp.							1	1		
Urocleidoides sp.					1	8				

The table summarizes the various parasites and fungi found in the animals. In contrast to bacteria, those agents are mainly specific to one species.

2 nonsexed). Seven (14%) cases were associated with *P. neurophilia*, which is a tad lower than expected (prevalence for *P. neurophilia* in the study is 18%—116 positives out of 612 analyzed). Conversely, *A. veronii/sobria* infections were three times as frequent (14%) in those fish as expected from the incidence on the whole sample (4.7%, 29 out of 612).

Husbandry practices

It is too early in this study to provide a full view of husbandry practices. A rapid summary can, however, be drawn (Table 3). Although all the staff in charge of animals were trained as required per law, few were specialists of aquatic husbandry. Out of the 23 structures visited, only 4 zebrafish facilities had staff initially trained as aquaculture specialist (6 out of 47). Those remaining were generally originally trained in biology, rodent experimental husbandry, or farming. Fourteen facilities had at least one technician dedicated to animal management, six facilities had two or more, and three facilities had no dedicated staff for the animal house.

Only 9 out of the 17 zebrafish facilities visited, but none of the *Xenopus* ($n=7$) facilities, had a separate quarantine room. Furthermore, two facilities housing both species had a dedicated quarantine room for zebrafish but none for *Xenopus*. Most *Xenopus* facilities used dedicated nonrecirculating tanks as quarantine as did zebrafish facilities lacking a quarantine room.

One half of the zebrafish facilities used a mixture of tap water and reverse osmosis (RO) water to adjust water hardness to the desired value, the other half used reconstituted water by dissolving artificial salt in RO water (Table 3). Of those, and following the Zebrafish Book recommendations,[33] seven out the eight facilities used Instant Ocean salt (Aquarium Systems), the last one used a freshwater salt: Preis Mineral Discus salt (Preis Aquaristik). The range of conductivity (measured in $\mu S \cdot cm^{-1}$) for *D. rerio* varied from 150 to 800 $\mu S \cdot cm^{-1}$. The conductivity was maintained in this range by the fish caretakers as those values were seen as close to the water quality prevailing during the natural mating season. Water temperatures were maintained from 24.4°C to 28.5°C with a median value of 27.4°C. All fish facilities used dry food pellets or flakes. Most of them (13 out of 17) used live *Artemia* nauplii at least for juveniles, frequently for adults, but few ($n=7$) used some live prey like rotifer, or paramecia because of the difficulty to maintain cultures.

For *Xenopus*, most facilities used either tap water (six facilities) and one used sea salt reconstituted water from RO water. The range of conductivity (measured in $\mu S \cdot cm^{-1}$) for *Xenopus* sp. varied from 450 to 3000 $\mu S \cdot cm^{-1}$. Water temperature in facilities housing *X. laevis* ranged from 18°C to 23°C, with a median value of 19.5°C. For *X. tropicalis*, the range of temperature varied from 24.1°C to 26°C, with a median value of 25°C (Table 3). Most facilities used nonrecirculating systems with no water flow, and water was changed once a week. Food distribution was limited to once or twice a week and sometimes using beef heart. The two facilities using techniques inspired from zebrafish with recirculating system and more regular feeding practices were more successful in raising *Xenopus*.

For all species, the most common chemical agent used as anesthetic was tricaine methane sulfonate (MS-222), but benzocaine, eugenol, and 2-phenoxy-ethanol were also used in a few facilities.

Discussion

In this article, we report a brief review of health monitoring performed in 23 aquatic research facilities between the end of 2012 and September 2015. Only 6 facilities had a scheduled health-monitoring program before our visit. In the other structures, veterinarians were called on irregular basis depending on the need (diseased animals, punctual peak mortality rate, or need for a health certificate). The RESAMA health-monitoring protocols were directly inspired by the protocol in place in the six previously monitored facilities. Among these facilities, 17 housed a total of 173,000 *D. rerio* and 4 housed *O. latipes* for 18,950 fish. Concerning amphibians, seven *X. laevis* and four *X. tropicalis* facilities hosted, respectively, 12,600 and 2,600 individuals (Table 1).

Most of the facilities had dedicated staff to care for the animals. In the smaller facilities, the laboratory technicians and researchers contributed to husbandry. Most of the staff we met were enthusiastic about their job. Quite many were initially or became aquarist after starting their job, but very few (12%) had received initial training in this field. It is, therefore, crucial to propose a continuing education session dedicated to those staff, a recurrent demand, and one of the actions the network needs to set up in the near future.

A majority of zebrafish facilities (9 out of 17) had a dedicated quarantine room. None of the *Xenopus* facilities did. Husbandry practices for the two species are very different, because most *Xenopus* facilities do not breed frogs in house, but order full-grown breeders regularly from one unique supplier or resource centre; relatively few different strains are maintained in a facility and direct interlaboratory exchanges are limited; in zebrafish, most facilities breed their stock in house, maintain a larger collection of transgenic/mutant lines, direct exchange between laboratories is frequent (mutants, transgenic lines), emphasizing the need for a dedicated room.

The most common chemical agent used as anesthetic was tricaine methane sulfonate (a.k.a. MS-222), and to a less extent eugenol, 2-phenoxy-ethanol. In France, none of these products get a national marketing authorization. Their use is, therefore, restricted to magisterial preparations made by a registered veterinarian or pharmacist. In Europe, benzocaine and tricaine used in overdose, is considered acceptable for fish and amphibian euthanasia (EC recommendations for euthanasia of laboratory animal[18]). 2-Phenoxy-ethanol and hypothermia are not acceptable methods.[34] 2-Phenoxy-ethanol is neurotoxic to humans and should be avoided for that reason. Hypothermia is a fish sedative, but has no analgesic properties. There is no EC recommendation for or against eugenol/isoeugenol. But, in our opinion, they are not advisable because of their low security margin in some species[34,35] and being inconvenient to users: they are allergenic[36] and carcinogenic,[37] often inducing arms dermatitis and headache in the fish farm environment (E.L., personal communication); in confined facilities, 1 person out of 10 complain about headache and dizziness after exposure to eugenol vapor (B.G., E.L., L.L., F.S., personal communications).

In French aquaculture, benzocaine solution is the main anesthetic used. It offers several advantages over tricaine. It is easier to handle (no buffering needed), its action is independent of pH and hardness. It is less expensive and more efficient than tricaine on fish and amphibians.[38–40] Benzocaine can be removed from aquaculture facility effluents using activated carbon filtration[41] and its breakdown time in water is about 4 h,[42] thus making this drug acceptable in terms of environmental contamination.[18] It is also useful in some species sensitive to MS-222 such as *Morone saxatilis*[43] and *Lampetra tridentata.*[39]

Most of the facilities have been visited once, except when routine health visits were compatible with the RESAMA protocol (same protocol, same visiting staff). In those cases, the results of each visit have been included in the database. In most zebrafish facilities, we sampled 30 animals or more (which is recommended to detect a pathogen present in 10% of the population with a confidence of 95%).[44] The size of *Xenopus* colonies did not allow such sampling, and 5–10 *X. laevis* and 3–5 *X. tropicalis* were sampled by facility. Indeed, the aim of this study was to increase awareness of caretakers and veterinarians around the specificity of aquatic species. The protocol used to sample the facilities was specifically chosen to maximize pathogenic species detection. We asked the facility managers to keep their routine and to avoid nonplanned weeding out of symptomatic fish before the visit. During the visit, symptomatic animals were preferentially chosen over asymptomatic animals, older animals over younger animals. The method is not suitable to perform an epidemiological analysis as it provides a distorted view of health in the facility. With an overall 30% of the sampled zebrafish positive for at least one pathogen (Table 2), the protocol obviously met its target. Indeed, in most facilities, we also collected nonsymptomatic animals. Those animals were largely disease free (3% of positives) and provide a more realistic view of the health status of the visited facilities, which were rather clean overall. Moreover, except *P. neurophilia*, no parasite was found or suspected in any zebrafish or medaka colonies. The only parasite found, *F. liguloides*, is a known parasite of *A. salina*. It was found exclusively in the digestive tract of zebrafish fed with frozen wild adult *Artemia.* The parasites were most probably dead and should be considered as an artifact (Table 5).

More significant, coinfections of zebrafish by *P. neurophilia* and *Mycobacterium* spp. were frequent. In the concerned facilities, both pathogens were prevalent. More likely, *P. neurophilia* infection may lead to increased susceptibility to secondary mycobacteriosis. More analysis will be required to get a real conclusive answer on this hypothesis.

Surprisingly, *Mycobacterium peregrinum*, *Mycobacterium haemophilum*, and *Mycobacterium abcessus* were not detected in any facility, which was unexpected in view of previous publications.[7,8,45,46] Similarly, the prevalence of mycobacteria in *Xenopus* facilities seemed to be very low. None of our visits took place during an epizootic outbreak when prevalence is high. Some authors[47,48] suggested that the *Mycobacterium* infection might be endemic and asymptomatic in wild *Xenopus*, showing clinical signs only after stressful stimuli such as transport, bad housing conditions, or sudden temperature drop. Our observations seemed to corroborate this hypothesis.

In zebrafish, we detected several species of mycobacteria. *M. chelonae*, which is the only *Mycobacterium* detected in the Zebrafish International Resource Center (ZIRC, Animal health report), was also the most frequently detected in the French research facilities. Indeed, the good efficiency of the biosecurity practices may have helped or the fact that the only available source of fish for research facilities came from registered suppliers, for example, limiting if not totally suppressing the risk to introduce parasitic agent, which are frequently found in pet shops.

In contrast, the *X. laevis* results showed many different kinds of parasites. *B. dendrobatidis* is suspected to be at least partly responsible for amphibian decline worldwide. *X. laevis* is a healthy carrier of this pathogen and is susceptible to release it in the wild. *A. hydrophila* has been reported to be the causative agent during epizootic outbreak in a *Xenopus* colony.[49] *A. hydrophila* seems to be able to jeopardize *Xenopus* innate bacterial defense by inhibiting the activity of the frog skin antimicrobial peptides.[50] The health profile of *X. laevis* remained closer to recently acclimated animals, despite their long presence in the laboratory. This may be explained by the use of animals from the wild and less efficient

biosecurity practices. However, life expectancy in captive *Xenopus* is long and the animals present in the facility are sometimes not more than two or three generations than wild caught/bred animals.

Noninfectious diseases were quite prevalent and diverse in all species. Some of them were distributed across the majority of the facilities, for example, steatosis in zebrafish facilities or gall bladder sludge in *Xenopus* facilities. In our opinion, those widespread diseases were mainly because of the current lack of knowledge about zebrafish exact diet requirements leading to an unbalanced feeding imperfectly compensated by a slight overfeeding. Others, such as nephrocalcinosis, liver neoplasia, and egg-binding, were more limited to some facilities. High incidences of those diseases are likely related to inappropriate husbandry practices. In trout, multiple factors may predispose to nephrocalcinosis, for example, poor water quality and high carbon dioxide levels[51]; imbalanced diet in particular magnesium deficiency[52]; and selenium toxicity.[53] Liver neoplasia and hepatomas and hepatic carcinomas are quite common in fish as liver is the preferential organ for detoxification. Improper fish feeds containing high levels of unsaturated fatty acid are prone to oxidation and aflatoxin production.[54] This is the most likely cause of liver neoplasia observed. To complete, egg-binding may be related to a lack of rigor in female breeding practice. However, egg-binding is frequently associated with microsporidia and/or mycobacteria infections. It remains, however, difficult which of the infections or the egg-binding is the causative agent.

To conclude, our results highlighted that a large number of the pathogens found in research facilities are common to most of the aquatic animals held there. These results support our approach to assess aquatic facilities as a whole. For facility managers, they emphasize the importance of sound sanitary barriers to be put in place in facilities housing multiple aquatic species.

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Author Contributions

B.G., L.L., and E.L. participated equally in this work. B.G., L.L., E.L., and F.S. designed the concept; E.M. has designed and written the database implementation and the website; B.G., L.L., E.L., and F.S. participated in the visits; E.L. performed necropsy with the help of B.G. and L.L.; L.M. and N.K. carried out the PCR and sequencing analysis; bacteriology was performed by N.K.; S.L. performed anatomo-pathology in coordination with E.L. All the authors participated in the analysis of the results; F.S. and E.L. wrote the article with the help of all the authors.

Disclosure Statement

No competing financial interests exist.

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