Vol. 117: 177–185, 2016
doi: 10.3354/dao02950

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published January 13

Germ-free sea bass *Dicentrarchus labrax* larval model: a valuable tool in the study of host–microbe interactions

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ABSTRACT: A thorough understanding of host–microbe interactions is crucial for more efficient disease management in the marine larviculture industry. As demonstrated in terrestrial animal research, gnotobiotic systems (involving animals cultured in germ-free conditions or inoculated with known microorganisms) are excellent tools to extend our understanding of the mechanisms involved in host–microbe interactions and allow the evaluation of new treatments for diseases. In this study, we introduce a germ-free European sea bass *Dicentrarchus labrax* larval model, independent of the continuous addition of antimicrobial agents. This model has an experimental set-up that allows addition of live feed to the larvae without compromising the germ-free status. This model will facilitate and render aquaculture research more effective in terms of mitigation fish larval diseases.

KEY WORDS: Aquaculture · Gnotobiotic · Pathogen · Larvae

INTRODUCTION

Food security is a pressing global concern as the world's population is estimated to reach between 8.5 and 10.5 billion by 2050. With capture fisheries becoming increasingly unsustainable due to overfishing, aquaculture is projected to overtake the former in supplying the world's marine protein requirements in the near future (FAO 2014). However, disease outbreaks impede the sustainable development of global aquaculture (Vadstein et al. 2013). Although treatment with antibiotics has been the main strategy for controlling infectious diseases, there is increasing acknowledgment of its limitations in terms of potential toxicity, the engenderment of resistance and residues, as well as environmental impairment (Alderman & Hastings 1998, Jones et al. 2004, Cabello 2006, Sapkota et al. 2008). As a result, there is a trend

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towards more strict regulations on the use of antibiotics in the aquaculture sector (Subasinghe et al. 2001, Romero et al. 2012), hence calling for alternative, sustainable methods to which the aquaculturist can resort for preventing and controlling disease outbreaks. Several environmentally friendly prophylactic disease treatments have been the focus of recent research, i.e. probiotics, prebiotics, vaccines, immunostimulants or antimicrobial peptides (Merrifield & Ringø 2014).

A judicious and scientifically supported application of the abovementioned alternatives warrants a thorough testing of their efficacy and safety under standardized and controlled experimental conditions (Smith et al. 2003). However, the stochastic colonization of larvae by microorganisms may hinder the establishment of a reproducible experimental set-up by generating high inter-individual and inter-batch variability in the composition of the standing microbial community. Hence, from a microbiological point of view, iterating experimental conditions using conventional animals is almost impossible (Fjellheim et al. 2012). The development of test systems in which the researcher has complete control over the microbial community structure, by adopting a germ-free or gnotobiotic model, was revolutionary in this respect. As already demonstrated in multiple terrestrial animal studies, gnotobiotic models are an excellent tool to extend our understanding of (1) the nutritional requirements of host organisms, (2) host–microbe interactions and (3) host metabolic functions (Gordon & Pesti 1971, Wostmann 1996, Marques et al. 2005). Despite the significant increase in the use of fish as experimental animals during the last decades (Marques et al. 2005, Schaeck et al. 2013) and the stressed importance of raising aquatic organisms gnotobiotically (Bates et al. 2006, Rekecki et al. 2009), the current know-how of rearing gnotobiotic aquatic organisms is much more limited compared to the more traditional mammalian laboratory animals. To our knowledge, only a handful of studies have reported the successful derivation of gnotobiotic marine fish species (Baker et al. 1942, Munro et al. 1995, Rawls et al. 2004, Dierckens et al. 2009, Forberg et al. 2011; for a review, see De Swaef et al. 2015). However, not all of these models included a system for axenically feeding the larvae, and some used only culture-based techniques to verify microbial status, with the potential of falsely claiming a germ-free status. Furthermore, all models relied upon a continuous addition of antimicrobial agents to the rearing water to safeguard the germ-free condition, with the exception of the zebrafish *Danio rerio* and Atlantic cod *Gadus morhua* models (Rawls et al. 2004, Forberg et al. 2011). The use of antimicrobial agents may elicit unwanted and unknown interactions with the target organism (Marques et al. 2006). Furthermore, the envisaged microorganisms need to be made resistant to the antimicrobial agent by multiple *in vitro* passages, which may have an impact on pathophysiological traits of the microorganism under study (Fux et al. 2005, De Swaef et al. 2015).

In this respect, the aim of our study was to develop a standardized germ-free sea bass larval model system, independent of the continuous addition of antibiotics and allowing germ-free feeding, which will facilitate the study of host–microbe interactions.

MATERIALS AND METHODS

All experiments were approved by the Ethical Committee of the Faculty of Veterinary Medicine and Bioscience-Engineering, Ghent University (no. EC2013/19). All procedures were conducted aseptically. Therefore, all materials and liquids were autoclaved (120°C, 20 min) or purchased sterile, and all manipulations were performed within a microbiological safety cabinet class II or a barrier isolator with a glove system (G(ISO)-T3, TCPS). Artificial autoclaved sea water (AASW, Instant Ocean®) and filtered (0.2 µm, Sartopore Pt MidiCaps, Sartorius) AASW (FAASW) were adjusted to a salinity of 33 ppt and a temperature of 16 ± 1°C.

Egg acquisition

Naturally spawned European sea bass eggs, aged 24–48 h post fertilization, were obtained from the 'Ecloserie Marine de Gravelines' (Gravelines, France). The viable, hence buoyant, eggs were skimmed off in the hatchery. Upon arrival, the eggs were collected on a nylon sieve (mesh size 300 µm) and gently rinsed with AASW (Salvesen & Vadstein 1995).

Short-term study

A short-term study was initially carried out employing 4 disinfecting agents to assess their capability to sterilize eggs and yield germ-free larvae at 1 d post-hatching (dph) with no marked adverse effects on hatching ratio. Four different disinfecting agents were tested, with varying concentrations and contact times: glutaraldehyde (Salvesen & Vadstein 1995, Dierckens et al. 2009), hydrogen peroxide, non-

thermal plasma (NTP; Jacobs et al. 2011, De Geyter & Morent 2012) and ozone, generated in dry air by a LAB2B ozone generator (Ozonia). All adopted protocols are listed in Table 2 (in the 'Results'). Hatching ratio for each incubation bottle was expressed using a semi-quantitative score: 3 (hatchability comparable with the eggs in the non-treated incubation bottles), 2 (hatching >50% of the control), 1 (hatching <50% of the control) and 0 (inability to hatch).

Long-term study

Approximately 200 eggs were transferred to a 50 ml Falcon (Greiner Bio-One) tube, filled with 40 ml of a 400 ppm glutaraldehyde solution (50 wt % solution in water; Merck), prepared with AASW. After 3 min of stirring, the eggs were transferred to a second 50 ml Falcon tube, again filled with 40 ml of a 400 ppm glutaraldehyde solution. After 3 min, the eggs were rinsed in 2 successive baths of AASW and were then collected and placed into 500 ml glass incubation bottles containing 400 ml of FAASW supplemented with a mix of antimicrobial agents (Table 1). The control eggs underwent identical physical handling as the disinfected eggs but with no glutaraldehyde or antimicrobial agents added to the (F)AASW. A low level of filtered (0.2 µm, Sartorius) air was provided to all incubation bottles. At 1 dph, the hatching ratio, the percentage of eggs hatched to total number of eggs was registered for every bottle. From each bottle, 24 larvae were stocked individually in sterile, polystyrene 24-well tissue culture plates (Greiner Bio-One), each well containing 2 ml of FAASW. The well plates were placed in a barrier isolator with a glove system and from then onwards, all procedures were performed inside the isolator. From 1 to 16 dph, 1 ml of FAASW was changed every other day. From 7 to 16 dph, larvae were fed live sterile *Artemia franciscana* nauplii (EG type, INVE Aquaculture) every other day, 20–30 per well (Sorgeloos et al. 1986). Larvae were subjected to a circadian rhythm of 8:16 h light:dark. Larvae were monitored daily until 16 dph, whereupon all larvae were euthanized by immersion in an overdose of MS222 (Sigma-Aldrich). This protocol was performed twice in time, adopting different egg batches. Each time and for both the non-disinfected and disinfected eggs, four 24-well plates were included.

Morphological analysis was performed to evaluate the impact of the germ-free conditions on larval growth and development. The standard body length was measured to the nearest 0.1 µm using an Olympus SZX7 stereomicroscope and cell D software (Soft imaging system, Olympus NV) and defined as the straight distance between the tip of the snout and the most caudal part of the larva. This was done for 3 larvae per 24-well plate of batch 1 at 0, 5, 9 and 16 dph.

Statistical analysis of the hatching and survival percentages were performed using a fixed effects model incorporating batch, treatment and their interaction in the model and comparing treatment with the residual variance (generalized block design analysis) at the 5% significance level. The length measurements were compared between the germ-free and conventional larvae by a mixed model with plate as random effect and day, treatment and their interaction as categorical fixed effects. With a significant interaction, the treatment was compared for each of the 4 days separately using Bonferroni's adjustment for multiple comparisons, i.e. using a comparison-wise significance level of 0.05/4 = 0.0125.

Evaluation of germ-free status

For the evaluation of the germ-free status of the larvae, water samples were retrieved from all incubation bottles at 1 dph for the short-term study, and from all well units of every 24-well plate at 7 and 16 dph for the long-term study. The water samples were inoculated onto marine agar and in TSB (+2% NaCl) and incubated at 17°C for 4 wk. In addition, a flow cytometer system was employed whereby water samples from the rearing water of 7 and 16 dph larvae were stained with a combination of SYBR Green I and pro-

Table 1. Antimicrobial agents that were included in the incubation bottles until hatching

Antimicrobial agent	Concentration (ppm)	Reference(s)
Ampicillin	10	Dierckens et al. (2009), Forberg et al. (2011), Situmorang et al. (2014)
Rifampicin	10	Dierckens et al. (2009), Forberg et al. (2011), Situmorang et al. (2014)
Penicillin	150	Munro et al. (1995)
Streptomycin	75	Munro et al. (1995)
Oxolinic acid	10	Munro et al. (1995)
Kanamycin	10	Munro et al. (1995), Rawls et al. (2004)
Erythromycin	10	Munro et al. (1995)

pidium iodide for microbial viability assessment. Samples were loaded on an Accuri C6 flow cytometer (BD Biosciences). Cell counts were determined by measuring the number of particles in a set volume (25 µl) after gating on green (FL-1) vs. red (FL-3) fluorescence plots in the BDC Sampler software (Swiss Federal Office of Public Health 2012, Van Nevel et al. 2013). FAASW was used to quantify the number of background particles.

RESULTS

Short-term study

The results of the various adopted disinfection protocols are listed in Table 2.

Eggs exposed to 2 successive rounds of 400 ppm glutaraldehyde treatment for 3 min resulted in germ-free larvae at 1 dph and equally high hatching scores as the control eggs.

Sea bass eggs showed strong resilience against high concentrations of H_2O_2, and germ-free eggs were consistently obtained after a 5 min exposure to 10% H_2O_2. However, upon working with these high concentrations, high inter-batch variability was observed in terms of hatching ratio.

Non-thermal dielectric barrier discharge and an atmospheric pressure plasma jet both resulted in 100% mortality of the sea bass eggs at a concentration that was still too low for rendering the eggs germ-free.

Ozone treatment was not able to consistently engender germ-free eggs without a marked effect on hatchability. Indeed, this treatment elicited a negative impact on hatchability upon employing the concentration and contact times needed to render the eggs germ-free.

Table 2. Disinfection protocols for European sea bass *Dicentrarchus labrax* eggs adopted in the short-term study, with their outcome in terms of hatching score (3: hatchability comparable to eggs in non-treated incubation bottles; 2: hatching >50% of controls; 1: hatching <50% of controls; 0: inability to hatch) and germ-free status of 1 d post hatching (dph) larvae; for assays with hatching score = 0, germ-free status was assessed using non-hatched eggs, 1 d after hatching of control eggs. GF/T: number of assays resulting in germ-free status/total number of assays for the protocol in question. DBD air: dielectric barrier discharge using air (Jacobs et al. 2011); APPJ argon: atmospheric pressure plasma jet using argon (Sarani et al. 2011)

Disinfection protocol		Assays (n) with hatching score				Germ-free status (GF/T)
Dose	Exposure time (min:s)	0	1	2	3	
Glutaraldehyde (ppm)						
150	03:00	–	–	3	–	0/3
400	03:00	–	–	–	1	0/1
400	03:00+03:00	–	–	–	12	12/12
800	03:00	2	–	–	–	2/2
Hydrogen peroxide (%)						
2	05:00	–	–	–	3	0/3
4	05:00	–	2	–	1	0/3
8	05:00	2	–	1	3	0/6
10	05:00	5	–	2	–	7/7
12	05:00	10	1	4	–	14/15
DBD air						
–	00:05	–	1	1	–	0/2
–	00:10	–	1	1	–	0/2
–	00:20	1	–	1	–	0/2
–	00:30	2	–	–	–	0/2
APPJ argon						
–	04:00	1	–	1	–	0/2
–	06:00	–	1	–	–	0/1
–	08:00	1	–	–	–	0/1
–	10:00	2	–	–	–	2/2
Ozone (mg l^{-1}_{gas})						
2	04:00	2	1	–	–	0/3
4	02:00	1	2	–	1	0/4
3	03:00	1	–	2	2	0/5
3	04:00	–	1	3	3	5/7
4	03:00	26	2	11	11	45/50
4	04:00	2	2	–	–	3/4

Long-term study

The hatching ratio of the disinfected and control eggs differed significantly ($p = 0.0122$), exhibiting a mean ± SE of 76.3 ± 1.6% and 68.7 ± 1.9%, respectively. The survival of the germ-free and control larvae also differed significantly ($p < 0.001$), exhibiting a mean of 94.3 ± 1.1% and 40.6 ± 1.1%, respectively.

From 9 dph onwards, standard length differed significantly between the conventional and germ-free larvae ($p < 0.001$), with the divergence rate increasing towards the end of the experiment (Fig. 1). By 16 dph, the germ-free larvae had a mean ± SD length of 5826.0 ± 90.4 µm, which was significantly higher than what was noted in the conventional larvae (5233.5 ± 107.2 µm).

Evaluation of germ-free status

Absence of bacterial growth on cultivated agar plates or turbid incubated broth indicated that none

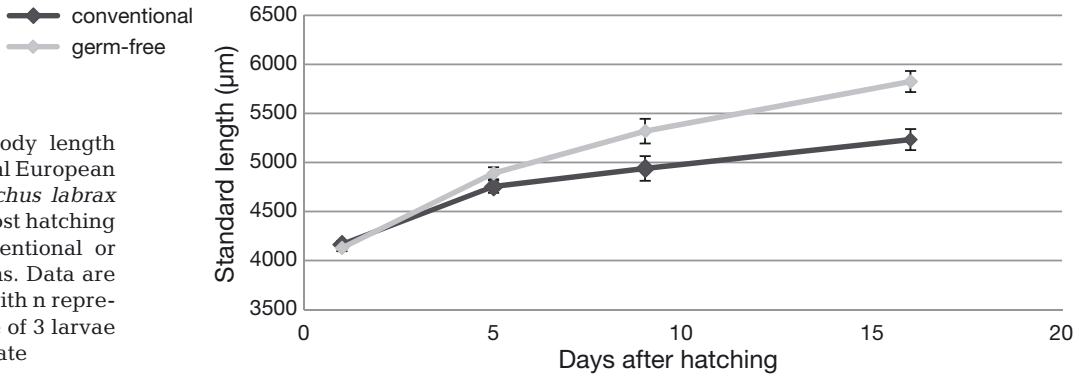

Fig. 1. Standard body length (mean ± SD) of larval European sea bass *Dicentrarchus labrax* at 1, 5, 9 and 16 d post hatching reared under conventional or germ-free conditions. Data are mean ± SD (n = 4) with n representing the average of 3 larvae per well plate

of the bottles or wells housing germ-free larvae harboured culturable bacterial cells. Flow cytometry was used to confirm the germ-free status, specifically targeting non-culturable bacterial cells. To eliminate the noise (background scatter) from the events highlighting bacteria, gates were set based on fluorescence (Fig. 2). Gate P1 included the particles that were detected in a water sample of conventional larvae and were not noted in an FAASW sample. The particles registered outside gate P1 were regarded as background noise.

DISCUSSION

The disinfectants glutaraldehyde, hydrogen peroxide and ozone were included in our experiments, because these were referred to in most other studies as valuable disinfectants for living tissue (De Swaef et al. 2015). In addition, non-thermal plasma, acknowledged as a promising disinfectant tool, was included (Moreau et al. 2008, Dobrynin et al. 2009).

Hydrogen peroxide, one of the most commonly used disinfectants in aquaculture, either for eggs, larvae or live prey, reacts as a strong oxidizing agent (McDonnell & Russell 1999). Up to now, only Douillet & Holt (1994) succeeded in obtaining sterile cultures of red drum *Sciaenops ocellatus* eggs after a 5 min exposure to 3% H_2O_2. An attempt to apply this protocol to other fish species failed in obtaining hatching eggs and germ-free larvae, indicating a high interspecies variability in sensitivity towards H_2O_2 (Douillet & Holt 1994). In our study, we observed strong resilience against concentrations as high as 10% H_2O_2. However, high inter-batch variability was observed in terms of hatching ratio. This could be related to the intrinsic egg quality, as working with suboptimal egg batches could exacerbate the negative effects on hatchability when adding an extra stress factor, such as high concentrations of disinfectant.

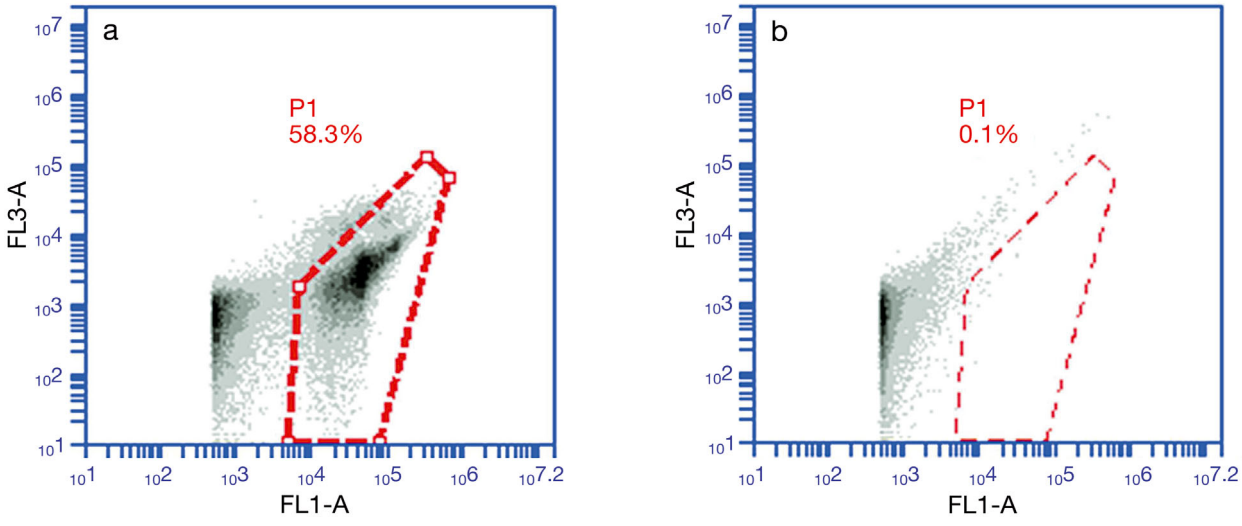

Fig. 2. Flow cytometer plots with gated events (marked area) to highlight bacterial contamination. Green fluorescence (FL1-A; *x*-axis) versus red fluorescence (FL3-A; *y*-axis). (a) Water sample (16 d post hatching, dph) from well housing conventional European sea bass *Dicentrarchus labrax* larvae. (b) Water sample (16 dph) from well housing germ-free sea bass larvae

Ozone, a fungicidal, bactericidal, virucidal and sporicial agent, has been successfully applied to obtain germ-free sea bass eggs, as well as gilthead sea bream *Sparus aurata*, common dentex *Dentex dentex* and red porgy *Pagrus pagrus* (Can et al. 2012). In our study, some egg batches showed great resilience to high ozone concentrations, although there was a high inter-batch variability in terms of hatching ratio. This may be a result of ozone dissolved in sea water which increases the resistance of the chorion protein polymer to hydrolytic breakdown, and/or inhibits enzyme secretion from the hatching gland (Ben-Atia et al. 2007).

Over the past years, NTP, which is in general any plasma that is not in thermodynamic equilibrium, has emerged as a novel tool in medicine (Moreau et al. 2008, Kong et al. 2009, De Geyter & Morent 2012), efficiently inactivating viruses (Yasuda et al. 2010), bacteria (Venezia et al. 2008, Kvam et al. 2012) and biomolecules such as DNA (Ptasińska et al. 2010). This inactivation occurs selectively, without burning the tissue, enabling the disinfection of living tissue without causing any damage (Fridman et al. 2006, Laroussi et al. 2012). In the present assay, 100% mortality was obtained at a concentration still too low for rendering germ-free eggs. This might be due to oxidative stress and damaged targeting enzymes, lipid membranes and DNA, caused by active short- and long-lived neutral atoms and molecules, generated by the NTP (Halliwell & Gutteridge 2007, Davies 1987).

Glutaraldehyde, known for its strong bactericidal, sporicidal and virucidal properties, retains a high level of activity in the presence of organic matter, which is generally present in batches of fish eggs (Fraise et al. 2008). The ability to destroy bacterial spores is an extraordinary property, making it the disinfectant of choice (Salvesen et al. 1997). Dierckens et al. (2009), as well as our short-term study, used only 1 round of disinfection with 400 ppm of glutaraldehyde for sea bass eggs (Salvesen & Vadstein 1995, Salvesen et al. 1997). We found this dose to be insufficient to produce germ-free sea bass larvae. We investigated the effect of repeating the glutaraldehyde disinfection process as was already demonstrated to be effective by Forberg et al. (2011), whereby germ-free cod larvae were obtained. Two consecutive rounds of glutaraldehyde disinfection, followed by a 48 h immersion in a mix of antimicrobial agents (Table 1), did not induce negative effects on the hatchability of the sea bass eggs and engendered a germ-free larval status until the end of monitoring at 16 dph.

As a result of multiple tests, a protocol was established to generate and rear germ-free sea bass larvae in 24-well plates until 16 dph. Compared to existing models, various plus points of this technique are evident, as outlined below.

Firstly, not administering antibiotics post-hatching offers the advantage of being able to include non-passaged bacterial isolates, thereby safeguarding the microorganism's original pathophysiological traits. The latter is important, as prior studies indicated that most acquired resistance mutations in bacteria confer a fitness cost (Melnyk et al. 2015). This may result in a longer generation time and altered metabolic activity, which are important determination factors for the establishment and the competitive capabilities of a strain in its microbial environment (Andersson & Levin 1999, Levin et al. 2000).

Secondly, the individual housing of the larvae in 24-well plates assures that the condition of an individual larva does not impact the other larvae. Indeed, Li et al. (2014) found that the mortality of gnotobiotic sea bass larvae challenged with *Vibrio anguillarum* was dependent on the number of dead fish in the vials at the moment of challenge. This indicates that individuals or their remnants can affect one another when housed together, explaining the high variability between replicates observed when working with group-housed larvae in challenge experiments. The successful rearing of sea bass larvae in a 96-well plate was already described by Panini et al. (2001). However, due to the small volume of the wells (200 µl) and the rapid proliferation of opportunistic bacteria in the rearing water and host, the larvae were only incubated successfully until 10 dph. In our survey, we used 24-well plates, allowing the water volume to be 10 times higher (2000 µl). In addition, larvae were reared under complete germ-free conditions. These adaptations allowed for successful rearing of the larvae until at least 16 dph. Survival rates of 95% were obtained, which are markedly higher compared to other experiments (e.g. Rekecki et al. 2009). We therefore hypothesize that larvae in 24-well plates will be able to survive for extended periods of time, considering the frequent water renewal, which prevented the accumulation of metabolites. The limiting factor when working with these microtitre plates will probably be related to the available space for larval growth. However, when research objectives would require prolonged maintaining of the larvae, 12- or 6-well plates may be implemented. In this respect, further research would indeed be very interesting for exploring the opportunities and boundaries of this model.

Thirdly, besides being used to study the effects of different treatments on larval growth, survival and physiological traits, the well-plate system is most promising to evaluate larval condition or behavioural characteristics. These are key components in the analysis of phenotypes resulting from genetic mutations, gene knockdown approaches, chemogenetic lesions, drugs and toxins. Motor function can be analysed for multiple individuals simultaneously by acquiring video images showing larvae swimming in the wells of multi-well plates (Ahmad et al. 2012). These automatic systems could additionally be applied to monitor general well-being of an individual larva, alerting the researcher as to which larvae exhibit a high range of behaviours cueing for anxiety, stress or (pre-) death which may give rise to the ability to identify humane endpoints. As most experiments performed with larvae have the potential to cause pain, suffering or distress, opportunities for refinement need to be explored and suitable humane endpoints identified (Schaeck et al. 2013). This individual housing system may offer a tool to initiate the pinpointing of humane endpoints in fish larval experiments.

A variety of methods has been described to monitor the germ-free animal status. Most prior studies working on gnotobiotic models relied solely on culture techniques, which are rather time consuming and may give false-negative results when dealing with slow-growing, or viable but non-culturable, organisms (Davis 2014). Therefore, non-culture based methods should be used additionally to support the claim that the animals are germ-free. Recently, flow cytometry has become an established and highly valued technique for the microbial analysis of aquatic samples (Díaz et al. 2010, Forberg et al. 2011, Prest et al. 2013). The greatest advantage of flow cytometry, besides speed, reproducibility and large sample sizes, is that the quantification and identification of organisms that formerly could not be detected by culture techniques is now achievable (Hernlem & Ravva 2007). In our model, we used culture-dependent techniques as well as flow cytometry, combining the best of both worlds and as such offering maximal certainty that this model effectively generates germ-free sea bass larvae.

The higher hatching and survival ratios of the germ-free larvae compared to conventional larvae are in accordance with other studies (Munro et al. 1995, Rekecki et al. 2009). The most obvious reason surface disinfection improves egg quality is that it prevents the proliferation of pathogens (Yoshimizu et al. 1995, Arimoto et al. 1996). In addition, fast proliferation of excessive amounts of bacteria on the egg surface may cause problems by reducing the exchange of gases and metabolic waste between the embryo and the environment (Vadstein et al. 1993, Salvesen & Vadstein 1995).

The morphometric data coincide with the findings of Rekecki et al. (2009). Andersen (2002) postulated different mechanisms that could explain these differences in growth rate: (1) the production of toxic metabolic by-products by resident gut bacteria; (2) competition of the microbiota with the host for energy and amino acids; and (3) inflammation caused by the commensal bacterial biota.

To our knowledge, this is the first disinfection protocol, independent of the continuous administration of antimicrobial agents in the larval rearing water that is able to generate germ-free sea bass larvae up to 16 dph. This tool opens up possibilities for extending our knowledge on the mechanisms involved in host–microbe interactions and to evaluate formulated diets and disease treatments in a standardized manner. We anticipate that this model could be adapted to develop germ-free husbandry protocols for other fish species. However, due to interspecies variability in egg size, chorion characteristics and optimal rearing temperatures in marine fish, the optimal treatment is likely to be species-specific, emphasizing the need for further refinement when other teleost species are envisaged.

Acknowledgements. We gratefully acknowledge Prof. Dr. P. Simoens for critically reviewing the manuscript. Furthermore, we thank Patrick Vervaet for transportation support; the Laboratory of Aquaculture & Artemia Reference Center (UGent) for their input; and Lore Vandermeersch (UGent) for help during the ozonation experiments. Finally, we acknowledge financial support from the Special Research Grant (Bijzonder Onderzoeksfonds, BOF12/GOA/022 and BOF12/BAS/070) of Ghent University, Belgium.

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Editorial responsibility: David Bruno, Aberdeen, UK

Submitted: June 22, 2015; Accepted: November 12, 2015
Proofs received from author(s): December 18, 2015