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Long-lived effects of administering β-glucans: Indications for trained immunity in fish

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ABSTRACT

Over the past decades, it has become evident that immune-modulation of fish with β-glucans, using injection, dietary or even immersion routes of administration, has stimulating but presumed short-lived effects on both intestinal and systemic immunity and can increase protection against a subsequent pathogenic challenge. Although the exact effects can be variable depending on, among others, fish species and administration route, the immune-stimulating effects of β-glucans on the immune system of fish appear to be universal. This review provides a condensed update of the most recent literature describing the effects of β-glucans on the teleost fish immune system. We shortly discuss possible mechanisms influencing immune-stimulation by β-glucans, including microbial composition of the gut, receptor recognition and downstream signalling. Of interest, in mammalian monocytes, β-glucans are potent inducers of trained immunity. First, we screened the literature for indications of this phenomenon in fish. Criteria that we applied include indications for at least one out of three features considered characteristic of trained immunity; (i) providing protection against a secondary infection in a T- and B-lymphocyte independent manner, (ii) conferring increased resistance upon re-infection and, (iii) relying on key roles for innate immune cell types such as natural killer cells and macrophages. We conclude that several indications exist that support the notion that the innate immune system of teleost fish can be trained. Second, we screened the literature for indications of long-lived effects on innate immunity of fish after administering β-glucans, a criterion which could help to identify key roles for macrophages on resistance to infection. We discuss whether β-glucans, as well-known immune-stimulants, are able to train the immune system of fish and argue in favour of further studies designed to specifically investigate this phenomenon in fish.

1. Introduction

Since the 1990's, when immune-stimulation of fish was still under early development, several investigations have suggested that the provision of β-glucans, either dietary or by supplementary injection, can potentiate the resilience of immune cells (Chen and Ainsworth, 1992; Jorgensen and Robertsen, 1995; Jorgensen et al., 1993), reduce in vivo stress effects (Chen and Ainsworth, 1992; Jeney et al., 1997) and protect teleost fish against subsequent challenges in vivo (Robertsen et al., 1990; Siwicki et al., 1994). Indeed, provided they are applied as a prophylactic measure, it has become evident that β-glucans can be a potential immuno-stimulant, suitable for injection and dietary administration, with well-described but short-lived effects on intestinal immunity, systemic immunity and increased protection from a subsequent pathogenic challenge (reviewed by (Dalmo and Bogwald, 2008)). Yet, detailed knowledge of the receptors involved in recognition of β-glucans and of their downstream signalling is missing for teleosts, leaving obscure whether the observed potentiation should be attributed to direct effects on leukocytes or to indirect effects on, for example, the composition of microbial communities in the gut. Typically, studies investigating the effects of β-glucans have mostly focussed on relatively short-lived effects, in the order of days up to a few weeks, but recent insights in the field of innate immunity provide indications that β-glucans could also have effects for a longer period of time, possibly explained by the phenomenon 'trained immunity' (see paragraph 4.3).

β-glucans are found not only in the cell wall of yeast species, including *Candida albicans* and yeast of the *Saccharomyces* genus (*Saccharomyces cerevisiae*, or baker's and brewer's yeast), but also in

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the cell wall of plants including wheat, rye and several *Echinacea* species, seaweeds, mushrooms and other fungi and even in the cell wall of several bacterial species (reviewed by (Meena et al., 2013)). In fact, β-glucans comprise a wide variety of structurally diverse molecules (Fig. 1), which can be short or long, linear or branched and have a soluble or insoluble character but which all have in common that they are polymers comprised of repeating units of glucose, linked by β-glycosidic bonds (Goodridge et al., 2009; Meena et al., 2013). Although β-glucans appear to have their immune-activating capacity in common, there can be clear differences in activity owing to the diversity in structure (Akramiene et al., 2007; Meena et al., 2013). In particular, large molecular weight β-glucans have stimulatory effects on leukocytes which include the induction of phagocytic, cytotoxic and antimicrobial activities (Meena et al., 2013). Probably the best-studied and most-applied β-glucans are large molecular weight *S. cerevisiae* and *Candida albicans* yeast-derived β-1,3/1,6-glucans.

2. Oral administration of β-glucans stimulates immune responses in fish

As mentioned above, β-glucans can be a potential immunostimulant for fish with clear effects, in particular, on innate immunity (Dalmo and Bogwald, 2008; Meena et al., 2013; Raa, 2015; Soltanian et al., 2009). These effects may not only depend on the branched structure but may also rely on the non-digestible nature of β-glucans (Raa, 2015). Non-digestible β-glucans may induce alterations in the composition of the gut microbiota and thereby indirectly influence the immune system (see paragraph 4.1) and/or the bacterial community in the gut may help to digest non-digestible oligosaccharides such as β-glucans into short-chain fatty acids with a physiological effect of their own (Swennen et al., 2006). Alternatively, the linear β-1,3 backbone ends up undigested in the proximal part of the intestine, where a proportion is phagocytosed by neutrophilic granulocytes and/or macrophages and degraded by a reactive oxygen species-driven process (Hino et al., 2012). Of interest, in salmonids, the uptake of laminaran, a linear β-1,3-glucan, via the posterior intestine results in a systemic accumulation in, among others, heart and spleen (Dalmo et al., 1994) whereas anal intubation with FITC-labelled yeast particles reveals uptake by mononuclear cells in the intestinal lumen (Lokka et al., 2014). The extent to which yeast and β-glucan particles are digested and/or taken up is still under debate, but it appears that the teleost intestine certainly is capable of uptake of β-glucans. The mechanisms behind antigen sampling and the cells involved in this process and present in the teleost gut are reviewed by Løkka and Koppang in this issue. We build on previous reviews of the subject and add a discussion of the more recent literature (2008–2015 in particular) grouped by (super)order, differentiating salmonids, perciforms and cyprinids based on the assumption that the closer the phylogenetic relationship the more reliable the conclusions. While also briefly discussing other routes of administration such as injection and immersion, the more practical route of oral administration through the diet will receive most attention.

2.1. Salmoniformes (salmonids)

One of the first studies on the protective effects of β-glucans, describing that intraperitoneal injection with a β-1,3/1,6 'M' glucan from *S. cerevisiae* enhanced resistance against two different bacterial pathogens, was performed in Atlantic salmon (Robertsen et al., 1990). It may not come as a surprise that subsequent studies addressing immune-modulating effects of β-glucans were performed in salmonids in particular. Based on the initial observations that β-glucans do indeed have immune-stimulating capacities, at least when injected, subsequent studies often included more practical routes such as oral administration (Table 1). The common picture that emerges from the studies on oral administration in salmonids is a confirmation of the immune-stimulating capacity of β-glucans, although with variable outcomes when it comes to increasing resistance against pathogens. For example, β-glucan treatment appears to increase resistance of Atlantic salmon to sea lice of the species *Lepeophtheirus salmonis* but not *Caligus elongates* (Refstie et al., 2010), for which β-glucan treatment may even lead to a higher infestation (Covello et al., 2012). Of course, β-glucans should not be considered miracle compounds able to increase resistance to all pathogens at all levels of infection.

Studies investigating the effects of β-glucans on maintaining the integrity of the gut have found no adverse effects and provide evidence for an assumed favourable increase in frequency of mucus-secreting cells in the epithelial barrier (Covello et al., 2012; Schmitt et al., 2015). Of interest, oral administration of rainbow trout with β-glucans appears to down-regulate the expression of immune-regulatory genes (e.g. IL-1β and lysozyme) in the presence of a microbial stimulus (Djordjevic et al., 2009; Skov et al., 2012), but up-regulate the expression of such genes (e.g. IL-1β and cathelicidins (host defense peptides)) in the absence of a microbial stimulus (Schmitt et al., 2015; Skov et al., 2012). These apparent contrasting effects of β-glucans on the expression of immune-regulatory genes, in the presence or absence of a microbial stimulus, could possibly help explain the variable outcomes with respect to increased resistance against pathogens mentioned above.

To verify and help explain the initial field observations, the number of laboratory-based studies aiming to acquire more detailed knowledge of the immune-stimulating effects of β-glucans in salmonids, have increased considerably. It has become clear that although the exact recognition receptors and downstream signalling routes still remain undefined, the immune-modulating effects of β-glucans on the immune system of salmonid fish should be considered stimulatory. Although the degree of disease protection offered by β-glucans clearly depends on, among others, the infectious agent, it should be noted that oral administration of β-glucans in salmonid species holds great potential as a prophylactic measure.

2.2. Perciformes (bass)

In Nile tilapia, β-glucans can rescue immune-compromised individuals treated with mercuric chloride, by feeding with a diet containing live *S. cerevisiae*, laminaran or purified β-glucans

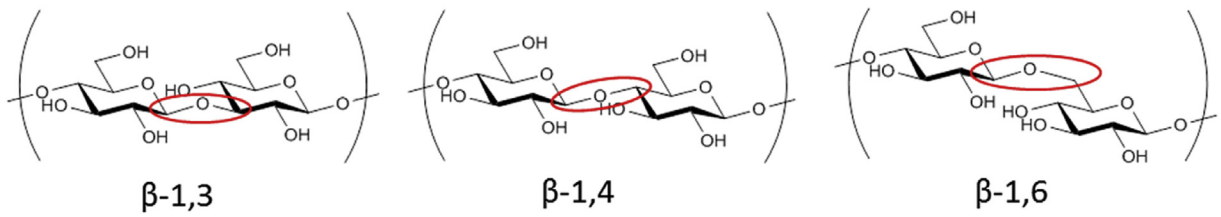

Fig. 1. Structure of β-glucan molecules. Examples of different linkages between repeating glucose units, determining the biochemical structure of diverse β-glucans.

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Table 1
Effects of oral administration of β-glucans in salmonid fish. Publications are grouped according to species, exact type of β-glucan used as immune stimulant and date of publication. The most pronounced outcomes of each study are summarized as "Results".

Species	Stimulant	Results	Reference
Rainbow trout (*Oncorhynchus mykiss*)	β-1,3/1,6-glucan (lentinan)	Decreased expression of pro-inflammatory genes in response to LPS (investigation by micro-array)	(Djordjevic et al., 2009)
Rainbow trout	β-1,3/1,6-glucan	Increasing trend in lysozyme activity (not significant) observed in glucan fed trout Glucan fed trout show increased resistance against challenge with *Ichthyophthirius multifiliis* (white spot) Trend visible after short term feeding, significant after feeding for a longer time period	(Lauridsen and Buchmann, 2010)
Atlantic salmon (*Salmo salar*)	β-1,3/1,6-glucan	No significant effect on diarrhoea-like conditions No implications on soybean meal induced enteritis No effect on number of sea lice (*Caligus elongatus*) infested fish or on number of sea lice per fish Significant lower salmon lice (*Lepeophteirus salmonis*) infestation (infested fish and lice per fish)	(Refstie et al., 2010)
Atlantic salmon	β-1,3/1,6-glucan	No effect on sea lice infestation, lice per fish even tends to be higher than control Histology showed no adverse effects of glucan enriched feed on intestines	(Covello et al., 2012)
Rainbow trout	β-1,3-glucan (*Euglena gracilis*) (≥98% purity)	No effect of β-glucan alone or as adjuvant on survival after *Yersinia ruckerii* challenge Down-regulation of expression of pro-inflammatory, acute phase and lysozyme related genes after challenge	(Skov et al., 2012)
Rainbow trout	β-1,3-glucan	Increased gene expression of cathelicidins 2 and IL-1β in gut epithelial cells Increased number of mucus secreting cells in the intestine	(Schmitt et al., 2015)

(from *S. cerevisiae*) (Table 2). The most pronounced effect was observed in fish fed with the purified β-glucans, where cellular and humoral immune parameters were restored to control levels and protection against subsequent challenge with *Aeromonas hydrophila* increased from 5% (control diet) to 60% survival (β-glucan enhanced diet) (El-Boshy et al., 2010). In most of the recent studies performed on bass species, oral administration of β-glucans not only increases innate immune parameters, such as phagocytic capacity and oxidative burst, lysozyme and complement activity (Chang et al., 2013; Dawood et al., 2015; El-Boshy et al., 2010; Guzman-Villanueva et al., 2014), but also increases protection against challenge with a number of bacterial pathogens including *Aeromonas hydrophila* and *Vibrio alginolyticus* (Chang et al., 2013; El-Boshy et al., 2010). In general, the observations on the immune-stimulating effects of β-glucans in perciforms (Table 2) support the findings in salmonids, with a comparable lack of mechanistic knowledge that could help explain recognition, signalling and immune-stimulation.

2.3. Cypriniformes (cyprinids)

Increasing attention has been given towards studying immune-modulating effects of β-glucans in cyprinids. As described above for salmonids and perciforms, also in cyprinids, oral administration of β-glucans stimulates a suite of innate immune parameters, again including stimulation of phagocytic capacity and oxidative burst, lysozyme and complement activity (Gopalakannan and Arul, 2010; Lin et al., 2011; Pionnier et al., 2013, 2014) (Table 3). Several studies in cyprinids have addressed the effect of β-glucan administration on (immune) gene expression (Falco et al., 2012, 2014; Miest et al., 2012; van der Marel et al., 2012). Among the investigated studies, continuous

Table 2
Effects of oral administration of β-glucans in perciform fish. Publications are grouped according to species, exact type of β-glucan used as immune stimulant and date of publication. The most pronounced outcomes of each study are summarized as "Results".

Species	Stimulant	Results	Reference
Nile tilapia (*Oreochromis niloticus*)	β-1,3/1,6-glucan or laminaran	Increased phagocytic activity and index in immunocompromised (IMC) fish due to mercuric chloride exposure Increased oxidative burst and neutrophil adhesion cells in IMC fish fed β-glucan or laminaran Increased survival after challenge with *Aeromonas hydrophila* in normal and IMC fish fed β-glucan	(El-Boshy et al., 2010)
Orange spotted grouper (*Epinephelus coioides*)	Mixture of β-1,4; β-1,3 and β-1,6-glucans	Increased lysozyme activity, alternative complement activation, phagocytic activity and oxidative burst Increased protection against *Vibrio alginolyticus* challenge	(Chang et al., 2013)
Gilthead seabream (*Sparus aurata*)	β-1,3/1,6-glucan (99% purity)	Increased IL-1β and IFNγ expression Increased phagocytosis and phagocytic index	(Guzman-Villanueva et al., 2014)
Red sea bream (*Pagrus major*)	Heat killed *Lactobacillus plantarum* (HKLP) in combination with commercial β-1,3/1,6-glucan	β-glucans significant increase the effect of HKLP, with respect to the lysozyme activity, the bactericidal effect, the alternative complement pathway activation and the total serum protein concentrations	(Dawood et al., 2015)

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Table 3
Effects of oral administration of β-glucans in cyprinid fish. Publications are grouped according to species, exact type of β-glucan used as immune stimulant and date of publication. The most pronounced outcomes of each study are summarized as "Results".

Species	Stimulant	Results	Reference
Common carp (*Cyprinus carpio*)	β-1,3-glucan	Increased oxidative burst Increased lysozyme activity Increased protection against *Aeromonas hydrophila* challenge	(Gopalakannan and Arul, 2010)
Koi carp (*Cyprinus carpio* koi)	β-1,3-glucan, Chitosan or Raffinose	Increased white blood cell count (WBC) Increased oxidative burst, lysozyme activity, phagocytosis, bactericidal effect	(Lin et al., 2011)
Common carp	β-1,3/1,6-glucan	Down-regulation of pro-inflammatory genes in gut and head kidney Decrease in IgM titer after *Aeromonas salmonicida* challenge (i.p injection) Increased expression of pro-inflammatory genes in head kidney after challenge but down-regulation in gut	(Falco et al., 2012)
Common carp	β-1,3/1,6-glucan	No apoptosis in head kidney cells Up-regulation of several anti- and pro-apoptotic genes Differential responses between different organs Upon LPS injection increased expression of pro-apoptotic genes in head kidney, rest of tested organs no effect	(Miest et al., 2012)
Common carp	β-1,3/1,6-glucan	Increased expression of β-defensin 1 and 2 and mucin5b in skin and β-defensin-2 in gills	(van der Marel et al., 2012)
Common carp	β-1,3/1,6-glucan	Increased basal CRP levels and alternative complement activation β-glucan augments the CRP and complement response to *Aeromonas salmonicida* challenge Differential effects observed between organs	(Pionnier et al., 2013)
Common carp	β-1,3/1,6-glucan	Reduced expression of immune-regulatory genes in the midgut (IL-1β, IL-10 and TNFα) Mx significantly increased upon poly(I:C) injection	(Falco et al., 2014)
Common carp	β-1,3/1,6-glucan	Increased serum complement activity Increased alternative complement activation	(Pionnier et al., 2014)

administration of β-glucans generally appears to result in an increased expression of pro-inflammatory genes, with a gradual decline over time depending on, among others, route of administration and immune organ under investigation (Falco et al., 2012; Pionnier et al., 2013). Continuous oral administration (25 days) of β-glucans can result in the up-regulation of anti-apoptotic genes in gut and head kidney, and of both anti- and pro-apoptotic genes in the spleen of common carp (Miest et al., 2012). The effects of β-glucans on apoptosis were further investigated and show that, in vitro, β-glucans can have a significant effect on apoptosis, but only at very high concentrations (Miest and Hoole, 2015). Taken together, these findings support the notion that oral administration of β-glucans may modulate the intestinal immune response and protect cyprinid fish from an acute (over)reaction (Falco et al., 2012, 2014). Since stimulation requires very high doses of β-glucans (500 μg/mL) to significantly increase apoptosis in head kidney leukocytes (Miest and Hoole, 2015), it does not appear that oral administration of β-glucans has major effects on programmed cell death of leukocytes. Strikingly, continuous oral administration of β-glucans up-regulates the expression of TLR3, a pattern recognition receptor assumed important for the recognition and binding of viral double-stranded RNA, leading to the subsequent triggering of a type-I interferon (IFN) response (Falco et al., 2014). The link between oral administration of β-glucans and up-regulation of a receptor for viral pathogen-associated molecular patterns such as dsRNA does not appear an obvious one and requires further investigation, before it can help to explain the mechanism behind protective effects of β-glucans on resistance against viral pathogens. Overall, it is becoming clear that oral administration of β-glucans stimulates the innate immune system of cyprinids as it stimulates the innate immune system of salmonid and perciform fish species, suggesting that the capacity to stimulate the innate immune system of fish is a capacity intrinsic to (large molecular weight) β-glucans.

3. Injection and immersion routes of administration of β-glucans

Although oral administration of β-glucans clearly is among the most practical applications, other routes of administration have also been investigated (Table 4). Maybe not always cost-effective, but intraperitoneal (i.p.) injection certainly is an effective method to deliver β-glucans and stimulate the immune system. For example, a single dose of β-glucans injected i.p. in rainbow trout resulted in a level of protection against infection with the microsporidian, *Loma salmonae,* similar to the level of protection induced by a 3 weeks feeding trial using 10 times higher concentrations of β-glucans. Interestingly, the effects of the single i.p. injection could be measured for a prolonged period of up to 9 weeks in vivo (Guselle et al., 2010) and up to 20 days ex vivo (no further time points measured) (Paredes et al., 2013). Protective and stimulating effects on innate immunity after i.p. injection with β-glucans have also been observed in zebrafish. A single i.p. injection 6 days prior

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Table 4
Effects of routes of β-glucan administration other than oral. Publications are grouped according to species, route of administration and date of publication. The most pronounced outcomes of each study are summarized as "Results".

Species	Stimulant	Route of administration	Results	Reference
Rainbow trout *(Oncorhynchus mykiss)*	β-1,3/1,6-glucan (*S. cerevisiae* or ProVale)	Intraperitoneal (i.p.) injection or oral administration	Hypertrophic lesion (xenoma) formation after *Loma salmonae* challenge reduced up to 92.5% (4 mg/mL) by i.p. injected research grade glucan Commercial glucan reduced xenoma formation by 64.5% (4 mg/mL) and 72.8% (10 mg/mL) High dose (20 mg/mL) abolished the reduction Oral administration of commercial glucan reduced xenoma formation dose dependently	(Guselle et al., 2010)
Atlantic salmon *(Salmo salar)*	β-1,3/1,6-glucan (laminaran)	i.p. injection dissolved in PBS or encapsulated in nanoparticles	Two days post injection, significant up-regulation in TNFα, IL-1β and IL-10 expression Encapsulated β-glucan invoked a stronger increase in IL-1β expression than unencapsulated glucans	(Fredriksen et al., 2011)
Atlantic salmon	β-1,3-glucan	i.p. injection	Macrophages show significantly increased oxidative burst Lysozyme and phagocytic activity at 10 and 20 days post injection (no further time point measured)	(Paredes et al., 2013)
Chum salmon *(Oncorhynchus keta)*	β-1,3/1,6-glucans	Treatment of eggs or gametes with glucan solution	Increased embryo and juvenile survival Increased resistance against *Saprolegnia* spp. infection	(Kiseleva et al., 2014)
Zebrafish *(Danio rerio)*	β-1,3/1,6-glucan	i.p. injection	Increased myelomonocytic cell counts Increased pro-inflammatory cytokine and chemokine expression Increased resistance against *Aeromonas hydrophila* challenge	(Rodriguez et al., 2009)
Common carp *(Cyprinus carpio)*	β-1,3/1,6-glucan	Bath immersion	Significant increase in wound healing Effects on the cytokine expression profile present but differential	(Przybylska-Diaz et al., 2013)

to challenge with *A. hydrophila* reduced the cumulative mortality, with similar although lower effects when injected only 2 days prior to challenge (Rodriguez et al., 2009). Increasing the frequency of injections further increased the survival of the zebrafish. Although the effects of even a single i.p. injection appear to be rapid and universal, only relatively few studies have addressed this route of administration, while this route could be of wider interest. For example, verification of immune-stimulation after injection of β-glucans could be of interest for injection vaccination protocols.

A potentially interesting alternative application of immune-stimulation induced by β-glucans is provided by the immersion treatment of fertilized eggs, or gametes, of chum salmon (*Oncorhynchus keta*) against infection with *Saprolegnia* spp.: a short treatment of 3 min only was sufficient to provide a significant protection against spontaneous infection with this oomycete (Kiseleva et al., 2014). This finding seems supported by the observation that both, pro- and anti-inflammatory genes were up-regulated after immersion of rainbow trout fry in a solution containing β-glucan (Zhang et al., 2009). Comparable to these studies in salmonids, there has been an increasing interest in applying β-glucans as immersion treatment to cyprinid fish. Although it appears difficult to convincingly show that β-glucan administration by immersion can (also) have a systemic rather than only a local effect on the immune system (Selvaraj et al., 2006), β-glucans can significantly improve wound healing of carp skin when applied to the water (Przybylska-Diaz et al., 2013). Mucosal organs, including the skin, typically are covered by a layer of mucus which is continuously renewed to prevent pathogen attachment and serves as a vehicle for antimicrobial compounds, complement, and immunoglobulins (Munang'andu et al., 2015). Administration of β-glucans by immersion, as modulators of mucosal surfaces of the skin or gills could be a promising new area of research, especially now that tools to reliably measure mucosal immunity are becoming available (Salinas, 2015). Possible explanations for immune-stimulating effects of β-glucan immersion baths could be sought, for example, in effects on the composition of microbial communities in the skin mucus (see also paragraph 4.1) (Lam and Chi-Keung Cheung, 2013) or increased local populations of alternatively–activated macrophages expressing a healing phenotype (Wiegertjes et al., 2016). Independent of the exact macrophage phenotype that would develop in the presence of β-glucans, given the most recent indications in humans that trained immunity can be stimulated via recognition of β-glucans by macrophages (see also paragraph 4.3), there is no doubt that modulation of macrophage function by β-glucans should remain an active area of research of fish immunology.

4. Mechanisms influencing immune-stimulation by β-glucans

Immune-modulatory effects of β-glucan administration have been widely observed and are generally considered as stimulatory for the health status of fish (Lam and Chi-Keung Cheung, 2013; Meena et al., 2013; Raa, 2015; Soltanian et al., 2009). One of the proposed modes of action indicates a prime role for the intestinal immune system, where induction of local intestinal inflammation

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after administration of β-glucans would result in a subsequent increased resistance against pathogens (Dalmo and Bogwald, 2008). A conclusive mode of action explaining the effects of β-glucans on the immune system of teleosts has yet to be uncovered.

4.1. Composition of microbial communities

The immune-stimulating effects of β-glucans not only depend on their branched structure but also rely on their non-digestible nature (Raa, 2015). Acid-treatment to mimic the effect of stomach-passage, completely abolishes the immune-stimulating effects of β-glucans on macrophages (Kudrenko et al., 2009). So far, this disruptive effect of low pH values has been reported in a single study only, but it could be important to further investigate to which extent (treatments mimicking) digestive processes can abolish the immune-stimulating capacity of β-glucans when administered orally.

Although beyond the direct scope of this review, one of the modes of action of oral administration of β-glucans could be to induce alterations in the composition of the gut microbiota. Feeding common carp with β-glucan-supplemented diets can modulate the microbial communities in the gut (Kuhlwein et al., 2013). Two weeks of feeding appears to reduce diversity, species richness and number of taxonomic units in the autochthonous (mucosal associated, indigenous) microbiota, a reduction not observed after 4 weeks of feeding. However, in another study, two weeks of feeding resulted in a clear increase rather than reduction in microbial community diversity, possibly explained by differences in samples, size of fish and analysis techniques (Jung-Schroers et al., 2015). In sea bass fed with β-glucans for 4 or 8 weeks, pyrosequencing of the intestinal microbiota revealed a transient alteration at the family taxonomic level in the composition of the autochthonous microbiota (Carda-Dieguez et al., 2014). It took a period of 4 weeks to completely shift the dominance within the microbial communities, which returned to the original composition after another 4 weeks of feeding. The data presented in these studies imply that effects of oral administration of β-glucans on the microbial composition in the gut are present, but could be transient and require further investigation. In line with these findings; the previously-mentioned effect of long-term feeding with β-glucans on TLR3 expression in the gut of carp could also be due to an indirect effect of β-glucans on the composition of the microbiota. In mice, a particular group of commensal bacteria present in the intestine have the ability to induce TLR3 expression, leading to the production of protective IFN-β (Kawashima et al., 2013). Given the immunological importance of the skin, especially in teleost fish (Gomez et al., 2013), it should also be of interest to study changes in composition of the microbial community of the skin after bath treatment with β-glucans.

4.2. Receptor recognition and signalling

β-glucans are thought to be internalized by phagocytosis, permitting their destruction by reactive oxygen and nitrogen species and by lytic enzymes in the acidic environment of the phagolysosome, largely based on information in mammals (reviewed by (Goodridge et al., 2009)). Among the best characterized phagocytic receptors are the opsonising Fc-γ receptor (FcγR) and the complement receptor 3 (CR3). Teleost fish do express genes encoding for the alpha- (CD11(b)-like) and beta-unit (CD18) of CR3 (Mikrou et al., 2009; Nakao et al., 2003), but at least zebrafish appear to miss classical members of the FcRs, although the genome does contain ancestral FcR-like genes (Akula et al., 2014). A distinct class A member of the Scavenger Receptor (SR) family, MARCO (macrophage receptor with collagenous structure), is present in several fish species, and knockdown experiments in zebrafish confirmed a role for MARCO in rapid phagocytosis of *Mycobacterium marinum* (Benard et al., 2014). In contrast, a clear role for the class B SR family member, CD36, could not be demonstrated in zebrafish and common carp (Fink et al., 2015). Toll-like receptors such as TLR2 could possibly sense β-glucans and are expressed in several fish species (Pietretti and Wiegertjes, 2014), but the presence of the prototypical C-type lectin receptor (CLR) for β-glucan, Dectin-1, is limited to mammalian genomes (Sattler et al., 2012).

In vitro modulation of fish leukocyte function by β-glucans is an active field of research that might help identify the exact receptor(s) involved and characterize (new) activation routes. For example, recent studies in common carp show that β-glucans can induce and increase robustness of Neutrophil Extracellular Traps (NETs) (Brogden et al., 2012, 2014). We studied the production of reactive oxygen and nitrogen species in response to β-glucans in common carp. Untreated zymosan and branched 1,3/1,6 β-glucans induce higher responses than zymosan depleted of TLR-stimulating activity and curdlan, both considered to be Dectin-1-specific ligands (Pietretti et al., 2013). The latter finding suggests recognition of β-glucans by multiple pattern recognition receptors on carp macrophages. Clearly, the relative importance of phagocytic receptors such as CR3, members of the SR and CLR superfamilies, and sensing receptors such as TLR2 with respect to β-glucan-stimulated immune responses has yet to be defined for fish. Identification of a C-type lectin receptor in fish with a role equivalent to the Dectin-1 receptor would be a crucial first step to subsequent studies of function based on, for example, the production of soluble receptors for β-glucan binding and carbohydrate competition assays.

4.3. Trained immunity

As mentioned earlier (paragraph 1), recent insights in the field of innate immunity provide indications that β-glucans could have effects over a longer timespan than initially anticipated, that might be explained by the phenomenon trained immunity. Traditionally, innate immune responses are characterized as rapid (hours-days), aspecific and without development of specific memory. In contrast, acquired immune responses are characterized as slow (days to weeks), highly specific (variable receptors on T and B lymphocytes can specifically recognize almost any antigen) and expressing long-term memory (based upon clonal expansion of memory T and/or B cells). Already, the classification of innate immune responses as aspecific has been challenged and thus, based on the presence of numerous classes of pattern recognition receptors (Akira et al., 2006; Janeway and Medzhitov, 2002) innate immunity no longer is classified as aspecific. At present, also the strict absence of a form of memory for innate immune responses is challenged by a new concept named trained immunity (Fig. 2), which is characterized by three criteria: (i) it can be induced after a primary infection or immunization and subsequently provide protection against a secondary infection in a T- and B-lymphocyte independent manner, (ii) it may be less specific than the adaptive immune response but still confers increased resistance upon reinfection of the host and, (iii) innate cell types such as macrophages and natural killer cells are key players in the mechanism, which involves improved pathogen recognition and an increased inflammatory response (Netea et al., 2011).

Of particular interest with respect to the present review, are observations in humans and mice on long-lived immune-modulatory effects of purified β-glucans and/or *C. albicans* on the development of trained immunity. In humans, pre-incubation of peripheral blood monocytes (PBMCs) in vitro with a single dose of *C. albicans* or β-glucans thereof, results in an increased cytokine production of, among others, IL-6 and TNFα, upon secondary

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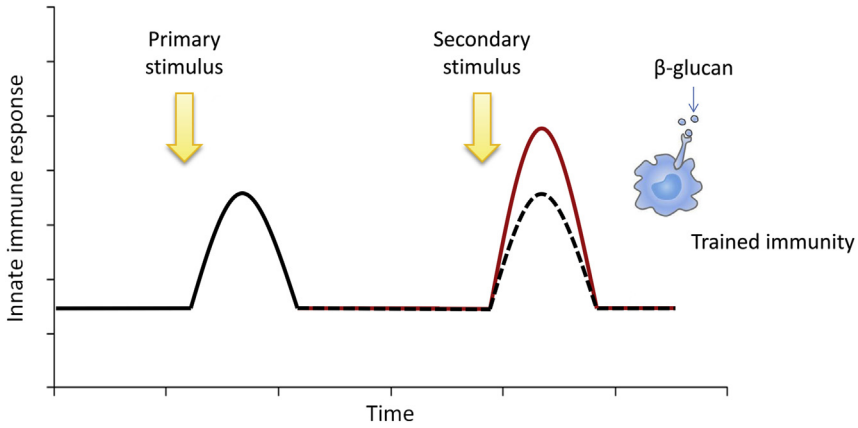

Fig. 2. The concept of trained immunity. Classical view on repeated stimulation of innate immunity (dotted black line) and updated view including trained immune responses (solid red line) (adapted from (Alvarez-Errico et al., 2015) and (Netea, 2013)). Arrows depict the moment of first and second stimulation with, for example, β-glucans. In the classical view, primary and secondary responses are of equal magnitude, whereas trained immunity results in a heightened secondary response facilitated by cells of the innate immune system.

stimulation with the same glucans for a relatively long period of up to 2 weeks (Quintin et al., 2012). Besides the increase in cytokine production, a metabolic shift is also observed; pre-incubated PBMCs have significantly higher glucose consumption and lactate production than control cells (Cheng et al., 2014). In mice, pre-treatment with highly purified β-glucans in vivo, protects animals from a subsequent infection with an unrelated pathogen, such as *Staphylococcus aureus* (Marakalala et al., 2013). The innate nature of this form of cross protection was investigated in recombination-activating gene (Rag) knock-out (KO) mice which lack functional B- and T-cells and thus lack functional adaptive immunity. The Rag-KO mice could be fully protected from a lethal infection with *C. albicans*, but only when pre-stimulated with a sub-lethal dose of *C. albicans* (Quintin et al., 2012), a phenomenon proposed to be monocyte-dependent. Despite clear in vitro effects, oral administration of β-glucans appears not to induce pronounced long-lived effects in vivo, in humans (Leentjens et al., 2014). However, administration of Bacillus Calmette–Guérin (BCG) does clearly induce trained immunity also in vivo. Effects induced by vaccination with BCG (Kleinnijenhuis et al., 2012, 2014), prepared from attenuated live *Mycobacterium bovis*, support the proposed benchmarks of trained immunity that it can elicit cross-specific protection in a T- and B-cell independent manner with innate immune cell types such as macrophages acting as key players (Netea et al., 2011). Of evolutionary interest, long before the recent discussions on the presence of trained immunity in humans and mice (Netea et al., 2015), similar cross-specific protection was observed in plants (Conrath, 2006; Ryals et al., 1996; Vernooij et al., 1995) and invertebrates (Kurtz, 2005) which, typically without T and B lymphocytes, can build up a form of immunity able to protect the organism upon a secondary exposure. Owing to the basal position of teleost fish as early vertebrates, it makes evolutionary sense to expect that trained immunity could be an important mechanism determining immune-stimulation of fish by β-glucans.

5. Future perspectives

5.1. Evidence for the presence of trained immunity in fish

Although teleost fish are among the evolutionarily oldest vertebrates with both an innate and classical adaptive immune system (Magor and Magor, 2001), there are several examples where innate immune parameters are more active and more diverse in fish than in mammals (Magnadottir, 2006). In line with these findings one would expect trained immunity should not only be present, but could have a pronounced role in the immune system of fish. There are at least a few studies providing preliminary evidence for the presence of a form of trained immunity in fish, primarily based on experiments with mycobacteria. Already in 1986, Olivier et al. observed a long-lived increase in phagocytic activity of peritoneal macrophages from Brook trout (*Salvelinus fontinalis*), for a period up to 33 days after i.p. injection with Modified Freund's Complete Adjuvant (MFCA) containing killed *Mycobacterium butyricum*. Only macrophages from trout injected with MFCA showed a significantly higher bactericidal activity (Olivier et al., 1986). Almost three decades later, the efficacy of injection with (modified) *Mycobacteria* was further investigated using BCG. Vaccination of Japanese flounder (*Paralichthys olivaceus*) with BCG resulted in an up-regulation of pro-inflammatory cytokines and conferred protection against *Mycobacterium* sp. (Kato et al., 2010). Also vaccination of Amberjack (*Seriola dumerili)* with BCG led to protection against challenge with *Mycobacterium* sp. (Kato et al., 2011). Importantly, these researchers could measure cross-specific protection, one of the proposed benchmarks of trained immunity. The cross-specific protection could be induced in Japanese flounder by BCG, shown by challenge with *Nocardia seriolae*, and was possibly mediated by bacteriolytic activity of the serum (Kato et al., 2012). The other benchmark of trained immunity; that cross-specific protection occurs in a T- and B-cell independent manner (Netea et al., 2011), was also studied in fish. Exposure of Rag-KO zebrafish to a sub-lethal infection with *Edwardsiella ictaluri* significantly protected the same animals from a subsequent lethal infection with the same bacteria. Crucially, protection could be transferred to naïve Rag-KO individuals by injection with kidney leukocytes from animals pre-exposed to the sub-lethal infection (Hohn and Petrie-Hanson, 2012). Together, these studies provide first indications for our hypothesis that trained immunity should be present in teleost fish and provide arguments that the innate immune system of fish can be trained by pre-exposure to sub-lethal pathogens. It remains to be investigated if trained immunity indeed has the predicted, pronounced role in the immune defense of fish and is indeed mediated by innate immune cell types such as macrophages.

5.2. Trained immunity induced by β-glucans: revisiting the literature

In mammalian monocytes, β-1,3-glucans purified from *C. albicans* are potent inducers of trained immunity (Cheng et al.,

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Table 5
Trained immunity-related effects of β-glucan administration in fish. Publications are presented according to order of discussion in the text. The column "Experimental set up" gives a concise summary of the parameters such as timing and route of administration. The most pronounced outcomes of each study are summarized as "Effects".

Species	Agent	Experimental set up	Effects	Reference
Zebrafish (*Danio rerio*)	β-1,3/1,6-glucan	Intraperitoneal (i.p.) injection 6, 4 or 2 days prior to bacterial challenge	Lowest cumulative mortality upon injection 6 days prior to the bacterial challenge	(Rodriguez et al., 2009)
Yellowtail (*Seriola quinqueradiata*)	β-1,3/1,6-glucan	i.p. injection on day 0 followed by a bacterial challenge on 15, 25, 35 or 45 days post injection	Minor increase in RPS (relative percentage survival) upon challenge on day 15 and 45 post injection	(Kawakami et al., 1998)
Blue gourami (*Trichopodus trichopterus*)	β-1,3-D-glucan (Laminarin)	i.p. injection followed by bacterial challenge 14 or 22 days post injection, or chemiluminescence assay 8, 14 and 22 days post injection	Increased oxidative burst in phagocytes up to 22 days post injection Increased survival upon challenge 22 days post injection	(Samuel et al., 1996)
Atlantic salmon (*Salmo salar*)	β-1,3-glucan	i.p. injection of β-glucan solution. Isolation of macrophages 10 and 20 days post injection	Significant increased oxidative burst, lysozyme and phagocytic activity at 10 and 20 days post injection	(Paredes et al., 2013)
Rainbow Trout (*Oncorhynchus mykiss*)	β-1,3/1,6-glucan	Oral administration of glucans. Subjects were fed control diet for 4 weeks, experimental diet for 2 weeks and finally 4 weeks of control diet	Glucans increase ConA induced proliferation of PBLs Glucans increase Ab titer in units/μL Increase in complement activation 8 weeks post vaccination (vaccination on week 0)	(Verlhac et al., 1998)
Sea Bass (*Dicentrarchus labrax*)	β-1,3/1,6-glucan	Oral administration of glucans. Subjects were sampled 4 weeks after the last feeding cycle	Increased serum complement activity and lysozyme activity	(Bagni et al., 2000)
Orange spotted grouper (*Epinephelus coioides*)	Mixture of β-1,4; β-1,3 and β-1, 6-glucans	Oral administration for 12 days followed by bacterial challenge at 0, 3, 6, 9, 12 and 15 days after switching back to control diet	Increased survival from bacterial challenge (up to 15 days after switching to control diet)	(Chang et al., 2013)

2014; Marakalala et al., 2013; Quintin et al., 2012). Until this moment, no studies have (knowingly) investigated induction of trained immunity in teleost fish in after administering β-glucans. After a first screen of the literature for clear indications of β-glucan-induced trained immunity in fish, we realized it was difficult to unambiguously ascribe observed effects to at least one of the characteristic criteria defined for trained immunity (paragraph 4.3 and (Netea et al., 2011)). Therefore, based on the assumption that innate immune responses generally are short-lived, we re-screened the literature for indications of long-lived effects on innate immunity after administering β-glucans (Table 5).

For example, a single i.p. injection of zebrafish (1–1.5 g body weight) with β-1,3/1,6-glucan provided partial protection against challenge with *A. hydrophila* at 6 days, but not at 2 or 4 days post-injection (Rodriguez et al., 2009). Similarly, but spanning a much longer time period, i.p. injection of yellowtail (*Seriola quinqueradiata*) with β-1,3/1,6-glucan induced increased resistance upon challenge with *Pasteurella piscicida* for a prolonged period of up to 45 days post-injection (Kawakami et al., 1998). Also, i.p. injection of Blue Gourami (*Trichopodus trichopterus*) with β-1,3-D-glucan (laminarin), induced increased resistance upon infection with *A. hydrophila* for a prolonged period of 22 days post-injection, which was the last time point measured. Of interest, the latter study provided insights into possible protective mechanisms by measuring an increase in phagocytosis-induced oxidative burst, suggestive of a key role for innate immune cell types such as macrophages (Samuel et al., 1996). Indeed, phagocytes could be responsible for long-lived effects induced by β-glucans since i.p. injection of Atlantic salmon with β-1,3-glucan leads to an increase in oxidative burst, phagocytosis and lysozyme activity of macrophages (Paredes et al., 2013). Most interesting in the present context; the latter study showed that increased macrophage activity was still measurable at 10-20 days post-injection, providing clear indications that single i.p. injections with β-glucans can induce long-lived effects in fish.

Continuous feeding with β-glucans for a number of subsequent days also appears to induce long-lived effects on the immune system of fish. For example, rainbow trout fed with β-1,3/1,6-glucans for a period of 2 weeks still showed higher antibody responses after vaccination against enteric redmouth disease and higher concanavalin A-induced proliferation of head kidney derived leukocytes 4 weeks after switching back to a control diet (Verlhac et al., 1998). Sea bass fed with β-1,3/1,6-glucan-enriched diets for 3 feeding cycles of 2 weeks followed by 10 weeks of control diet, showed higher serum complement and lysozyme activity than fish fed a control diet only (Bagni et al., 2000). Grouper fed a diet containing a mixture of mushroom-derived β-1,4; β-1,3 and β-1,6-glucans for a continuous period of 12 days still showed higher protection against challenge with *Vibrio alginolyticus* 15 days after switching back to a control diet (Chang et al., 2013).

6. Concluding remarks

The studies discussed here provide indications in existing literature for long-lived effects stimulated by β-glucans, possibly based on key roles for macrophages. There also are several studies that support the notion that the innate immune system of teleost fish can be trained. Whether β-glucan administration, either by injection, bath or oral route, indeed triggers trained immunity in a manner similar to what is observed for humans and mice requires more detailed studies specifically designed to investigate the phenomenon of trained immunity in fish. For example, it could be of interest to analyse, in detail, long-lived effects of stimulation with β-glucans in *in vitro* models based on purified populations of innate immune cell types, among which macrophages. Such studies would provide fundamental knowledge on mechanisms basal to trained immunity and conserved in cold- and warm-blooded vertebrates.

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