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Decoding the Black Box of Fish Vaccines Efficacy in Basic and Applied Contexts

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ABSTRACT

Vaccines are the frontline defense in aquaculture, yet predicting their real-world performance remains a tricky puzzle. Unlike terrestrial veterinary vaccines, fish vaccines largely operate through "Black Box" processes, where we observe the outcomes but rarely understand the underlying immune mechanisms. Laboratory trials, though informative, often fail to capture the complexity of commercial farms, where environmental fluctuations, pathogen diversity, host genetics, and stressors interact. Field studies provide glimpses into vaccine performance, but they are sparse, short-term, and sometimes biased, leaving many questions unanswered. Environmental stressors, particularly reuse water, low oxygen, ammonia accumulation, and fluctuating temperatures, can undermine even well-designed vaccines. Handling, vaccination stress, and the developmental stage of the fish further influence immunity, explaining why the same vaccine may succeed in one setting and fail in another. Immunologically, vaccine success depends on effective antigen processing, a balanced mix of systemic and mucosal responses, and durable memory, yet these factors are rarely measured in the field. Mucosal vaccines show promise by targeting pathogen entry points, while systemic responses remain essential for broad protection. This review explores the "Black Box" nature of fish vaccines, in which vaccination inputs and protective outcomes are observable, yet the internal interactions among environmental factors, host biology, and immune mechanisms remain poorly defined. Unlocking these factors is key to turning unpredictable vaccines into reliable tools for sustainable disease control in a growing aquaculture industry.

1 | Introduction

Aquaculture is expanding at an unprecedented pace, and vaccines have become one of its most critical tools for disease management. The global fish vaccine market, currently valued at around USD 410 million, continues to grow by nearly 10% annually, reflecting both technological progress and increasing demand for sustainable aquaculture solutions [1]. As the aquaculture vaccine market continues to grow rapidly and deliver high returns on investment, vaccines are increasingly relied upon as primary disease-control tools; however, the real-world performance of many fish vaccines remains difficult to predict. Unlike veterinary vaccines for terrestrial animals, which benefit from standardized correlates of protection (CoPs) and long-term field monitoring [2–4], fish vaccines largely operate within a non-transparent evaluation system [5]. Products are licensed and deployed primarily based on survival endpoints [6], while the underlying mechanisms determining success or failure remain largely uncharacterized. Laboratory trials, though essential, rarely capture the environmental complexity, host heterogeneity, and stressors that fish encounter in commercial production. Consequently, vaccines that appear highly effective in controlled experiments often provide inconsistent or suboptimal protection on farms [6–8]. If we borrow the analogy of "Black Box" and "White Box" concept from software testing, fish vaccinology presents

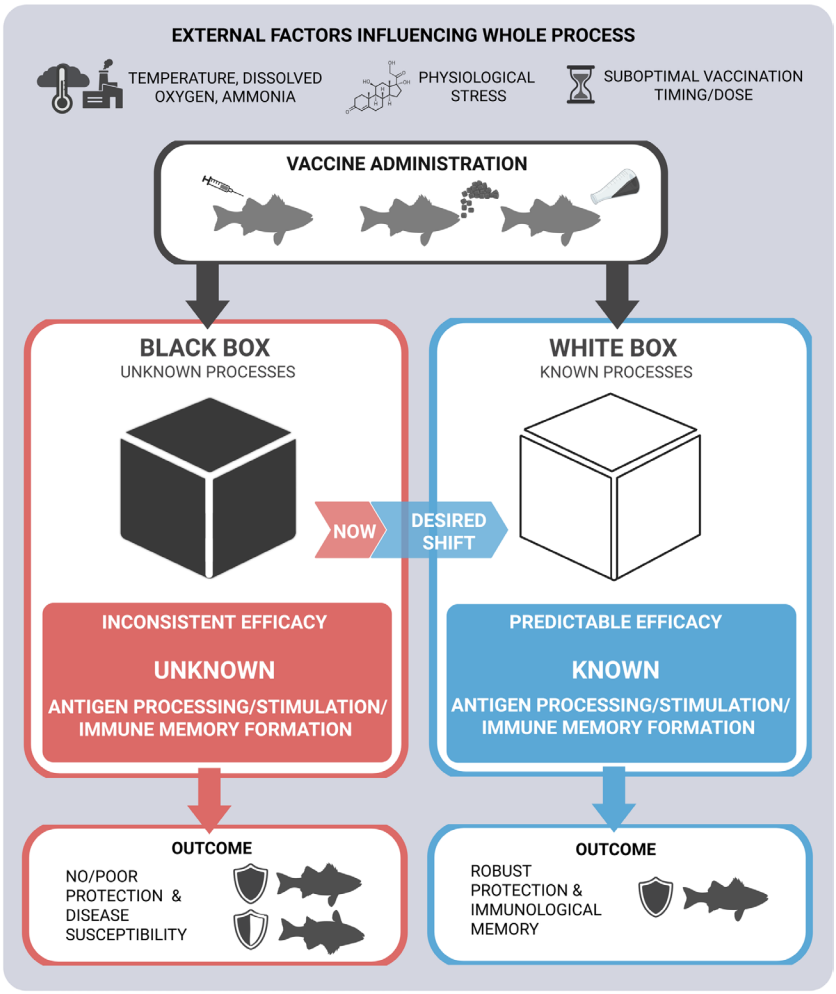

FIGURE 1 | Conceptual framework contrasting the current "Black Box" model, in which external stressors and unknown processes lead to inconsistent vaccine efficacy, with the desired "White Box" model, characterized by robust and predictable protection driven by known, well-defined processes.

a similar conceptual dilemma (Figure 1). In this framework, the "Box" represents the complex network of internal and external processes that influence and govern the organism (fish). The extent to which these interconnected processes can be observed, interpreted, and mechanistically resolved determines whether vaccine responses are approached as a "Black Box" or a "White Box" testing [9, 10]. In the context of this review, however, we focus on only one segment of this broader "Box," namely, the immunological and physiological processes activated following vaccination. The complexity and limited mechanistic understanding of these interconnected responses are what primarily constitute the "Black Box" in fish vaccinology (Figure 1). We observe only the inputs (vaccination) and outputs (survival or protection), while the mechanisms operating within the fish remain obscure. Crucially, this internal code is not a purely systemic script. It is a multilayered dialogue initiated at mucosal interfaces followed by machinery of programs within both systemic and mucosal immune axes. By contrast, a "White Box" system is defined by a fully traceable and predictable architecture of the whole organism processes (Figure 1). In this case, a subset of the internal processes governing how the fish interacts with the vaccine are transparent and mechanistically understood. Such a framework moves vaccinology beyond empirical application toward a system of biological transparency, enabling vaccine-induced pathways to be tracked in exquisite detail. However, achieving such a near-perfect system requires several critical catalysts to accelerate the development of effective vaccines. In addition, "White Box" system requires multilayered components, including standardized immunization guidelines tailored to species-specific ontogeny and stress mitigation, predictive efficacy trajectories mapping immune kinetics across fluctuating environmental landscapes, and quantifiable CoPs that statistically bridge individual immune markers with population-level outcomes. Currently, our understanding of fish vaccines still falls within the latter "Black Box" category, where the underlying pathways determining protection or failure are rarely accessible or systematically monitored. This scenario arises from a combination of factors: fish are ectothermic and genetically diverse; water temperature, quality, and microbiota fluctuate, pathogen strains vary regionally, immune processes are not fully defined, and vaccination timing often intersects with periods of immunological immaturity or stress. Together, these variables obscure the link between immune mechanisms and observed outcomes. As a result, predicting field efficacy from laboratory data remains

challenging. The route from antigen detection and processing to effective immune responses is also more complicated than we thought. Understanding this complexity is now becoming critical. Vaccination remains the safest, most environmentally friendly approach to preventing disease outbreaks in aquaculture, outperforming antibiotics or chemical interventions in terms of sustainability and animal welfare. Investment in fish immunovaccinology ranging from molecular studies of antigen processing to multi-season field trials directly supports food security, farm productivity, and animal health. Although fish vaccines in aquaculture remain a puzzle, with many mechanistic and efficacy aspects unresolved, unlocking their full potential to make them "White Box"-observable agents promises transformative productivity outcomes. Advancing our mechanistic understanding could transform fish vaccinology, enabling more predictable and effective disease control in aquaculture.

2 | Fish Vaccines: From Laboratory Efficacy to Farm Reality

Translating advances in fish immunology and vaccinology from controlled laboratory settings to commercial farms remains one of aquaculture's most persistent challenges. Laboratory challenge models rarely replicate natural exposure dynamics, and fish are often vaccinated during periods of immunological immaturity or physiological stress. Environmental variability, stocking density, pathogen diversity, and management practices differ dramatically between experimental tanks and production ponds. Together, these factors affect not only vaccine performance itself but also how reliably that performance can be evaluated across farms and production systems. As a result, vaccines that demonstrate strong efficacy under laboratory conditions may underperform in real-world settings, exposing the concealed nature of fish vaccines, where performance under experimental and commercial conditions often diverges. Since the early 1990s, only a limited number of field studies have rigorously assessed bacterial and viral vaccines, leaving much of the evidence for commercial performance confined to industry data with limited independent verification (Figure 2). Delivery method further complicates translation. Most field trials continue to rely on intraperitoneal (IP) injection, although immersion and oral routes are increasingly tested, particularly for bacterial vaccines (Figure 2A). Unlike IP injection, immersion and oral vaccination primarily engage the mucosal immune system at the gill, skin, and gastrointestinal surfaces, where antigen uptake, local antibody production, and epithelial barrier integrity collectively determine vaccine performance [11–13]. Consequently, variability in the efficacy of these delivery routes is not only a function of antigen formulation but also of the physiological and immunological state of mucosal tissues at the time of exposure [12, 14]. Therefore, IP injection remains the most common method with a high median relative percent survival (RPS); recent oral and immersion trials (particularly for bacterial pathogens) are showing competitive efficacy rates, suggesting viable alternatives to injection (Figure 2A). Clustering of high-RPS outcomes (> 90%) in the post-2020 period, driven largely by optimized IP formulations alongside the broader adoption of mucosal vaccine modalities, including immersion and oral delivery (Figure 2B) [15]. Together, these developments indicate a gradual narrowing of the performance gap between mucosal and injection-based vaccination strategies. Several strategies have been developed to improve vaccine protection, particularly for oral and mucosal delivery systems. Oral vaccines reached high efficacy by antigen encapsulation techniques that protect antigens from gastrointestinal degradation, as well as the incorporation of mucosal adjuvants that improve antigen uptake and local immune activation [13, 16–18]. Additional delivery-based approaches, including mucoadhesive formulations, nanoparticle carriers, and pre-treatment strategies such as hyperosmotic immersion, showed an increase of antigen retention and uptake at mucosal surfaces [19–23]. In parallel, improvements in protective antigen selection are increasingly driven by computational and systems-level approaches. Reverse vaccinology and immunoinformatic-based epitope prediction [24–27] now enable more targeted identification of immunogenic candidates compared to traditional empirical screening methods [28], improving the likelihood of selecting protective antigens with broad efficacy. Despite these efforts, assembling a comprehensive understanding of how vaccines perform under farm conditions is hindered by irregular reporting, variable study design, and a paucity of multi-season, long-term trials. A critical component of such monitoring is systematic documentation of vaccine usage [29], including vaccination status, which allows evaluation of both protective efficacy and potential side effects [30]. This has prompted renewed efforts to improve field trial design, standardize reporting, and enhance post-licensure monitoring, reflecting a broader recognition of existing limitations.

2.1 | What Field Trials Really Tell Us and What They Do Not

Although relatively low in number, well-documented field studies offer valuable insights into the real-world performance of fish vaccines. Many published trials report moderate-to-high protection against bacterial [31–39] and viral pathogens [40, 41], yet several vaccines remain unvalidated under farm conditions, and others show marginal efficacy outside the laboratory (Figure 2; Table S1) [42–45]. Direct comparisons of multiple commercial or experimental vaccines within the same field trial remain rare. As a result, our understanding of relative vaccine performance is fragmented and sometimes leads to a contradictory picture of overall performance [32, 46–48]. This partial perspective is further compounded by several structural blind spots that limit the conclusions field trials can lead to, which can be broadly grouped into issues of (i) publication bias, (ii) limited immunological resolution, and (iii) ecological complexity. First, the available literature is almost certainly shaped by publication bias. The trials reporting weak or negative field performance are less likely to be published, meaning the scientific record is likely to overrepresent successful outcomes. This skew further deepens the "Black Box" segment, when we observe only the more favorable outcomes, not the full spectrum of field experiences. Second, even when field trials are available, their immunological depth is often insufficient. Many studies focus on survival or gross pathology but lack detailed measures such as antigen-specific antibody kinetics, cellular immune responses, or mucosal markers.

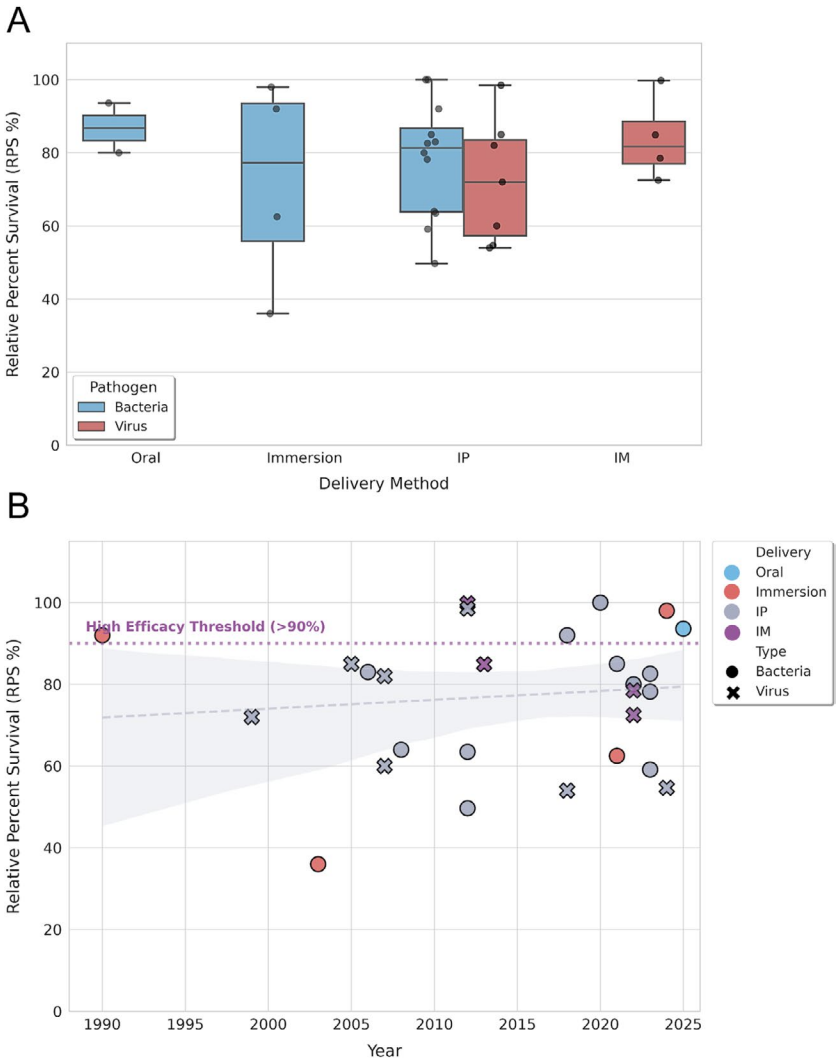

FIGURE 2 | Comparative analysis of fish vaccine field trial efficacy (1990–2025). (A) Efficacy distribution across different vaccine delivery methods. (B) Temporal trends in vaccine field trials. Clustering of high-RPS outcomes (> 90%) showing a positive trend in vaccine development efficacy over the last decade. IP: intraperitoneal delivery; IM: intramuscular delivery. Plots were analyzed and generated using the exploratory data analysis (EDA) packages (Pandas, NumPy, and Seaborn). Relative percentage survival (RPS) values presented as ranges in the source table were calculated as the means for plotting, while trials lacking quantitative data were excluded. Data were retrieved from the list of studies provided in Table S1.

For example, while an ELISA-based potency test for *Aeromonas salmonicida* vaccines can correlate serum antibody levels with protection in challenge trials, such immunological monitoring is only marginally incorporated into field evaluations, where predictive biomarkers are most urgently needed [49]. Moreover, in an open-sea cage study of *Piscirickettsia salmonis* vaccine, only short-term upregulation of innate and adaptive genes has been documented, without long-term follow-up of adaptive immune memory [50]. As a result, we often observe field outcomes without understanding the immunological pathways that produced them. Third, pathogen and environmental heterogeneity add layers of complexity that challenge vaccine efficacy in ways laboratory conditions cannot replicate. Differences in circulating strains, co-infections, seasonal stressors, and production practices create ecological noise that remains largely unmapped in most field evaluations. A striking example is again *P. salmonis* vaccination showing that two commercial vaccines provided virtually no protection against two prevalent genogroups in cohabitation trials that more closely mimic natural infection [7]. A review on vaccine failure in salmon also highlights that the mismatch between vaccine and field strains, co-infections, environmental stressors (low oxygen, high temperature), and genetic variability of the host can all contribute to field vaccine failure [7, 51–53]. This situation underscores the need for fish immunology research to provide deeper mechanistic insight into vaccine-induced protection, an effort reflected in recent studies seeking more detailed molecular understanding [54]. These limitations become even more evident when considering vaccines against eukaryotic parasites. There are only a few candidates that have undergone preliminary field testing, with limited success [55, 56], and one longstanding commercial vaccine against sea lice available [57]. The complexity of parasite life cycles and antigenic variability prevents a single universal vaccine, highlighting the limitations of current empirical approaches. Altogether, these points reinforce a troubling conclusion. The field trials may reveal some outcomes, but they rarely expose the mechanistic basis for protection or failure. Without broad and transparent immunological monitoring, and without accounting for

ecological and pathogen variability, vaccines remain "Black Box" operating tools, their internal code is hidden, and our capacity to predict or improve their performance in farms or under field conditions more broadly is severely limited.

2.2 | Measuring Efficacy in Real World: A System Not Built for Clarity

Assessing vaccine efficacy in aquaculture is inherently more complex than demonstrating survival in a laboratory challenge, reflecting methodological and conceptual limitations in how efficacy is currently defined and measured. Short-term trials often fail to capture long-term immunity, seasonal variation, or repeated pathogen exposure. For instance, protection shown in controlled challenge experiments may wane or change when the fish face fluctuating environmental conditions or diverse pathogen pressures over production cycles [47, 58, 59]. The dominant metric in field and laboratory trials, RPS, has clear limitations. While it is simple and widely used, RPS can be extremely sensitive to baseline. Alternative epidemiological measures such as relative risk reduction (RRR), absolute risk reduction (ARR), or number needed to treat (NNT) could offer more realistic estimates of vaccine benefit, particularly in high-density production systems where modest absolute risk reductions can translate into large population-level and economic effects but remain rarely used in aquaculture studies [51]. This neglect is a missed opportunity to quantify risk more meaningfully. Indeed, in a recent review on *P. salmonis* vaccines, it was argued that ARR and NNT should complement RPS because they provide a more accurate picture of how many fish benefit from vaccination. However, limitations in outcome metrics are only part of the problem. Beyond metrics, perhaps the most challenging gap is the lack of validated correlates of protection (CoPs) in fish. As noted in the literature, fish vaccine development remains hindered by limited mechanistic understanding. Although antibodies (especially IgM) are often measured, their levels do not always predict protection [5]. For example, in Nile tilapia (*Oreochromis niloticus*) vaccinated against *Streptococcus agalactiae*, where IgM titers drop while relative survival remains relatively high, suggesting involvement of additional immune mechanisms [60]. IgM titers completely lack predictive power when immunity is based on virus-specific cytotoxic T cells (CTLs), as is the case with some vaccines against salmonid alphavirus (SAV), where protection correlates with significantly stronger and faster specific cytotoxicity only post-challenge [61]. This emphasizes that, unlike human or terrestrial veterinary vaccines [62], aquaculture vaccinology still lacks well-defined immune biomarkers that are reliably predictive of protection. This limitation is not only technical but also reflects a broader challenge in vaccine development. In human vaccinology, the lack of well-defined CoPs is a major obstacle to advancing vaccine candidates into late-stage development, especially when standard efficacy trials are difficult to conduct due to low disease incidence, logistical challenges, or unpredictable outbreaks [63]. Similar challenges occur in aquaculture, where field trials are often complicated by environmental variability, pathogen diversity, and differences between production systems. As a result, vaccine performance is frequently assessed using large-scale survival endpoints or relative percent survival (RPS). However, these measures are comparable to relying solely on late-stage clinical outcomes without supporting mechanistic biomarkers, which reduces predictive accuracy and slows the optimization of vaccines. The absence of well-validated CoPs contributes directly to our borrowed analogy of the "Black Box" problem. Thus, despite the availability of survival or RPS data, the absence of mechanistic biomarkers prevents reliable prediction of which vaccines will perform well under commercial conditions, or why some batches or strains underperform. In this context, outcome measures can be viewed along a continuum of mechanistic insight. At one end are downstream phenotypic outcomes such as mortality and relative percent survival (RPS). These are followed by intermediate indicators, including pathogen load and tissue pathology. At the most informative end are proximal immune correlates, which directly reflect the mechanisms of protection. In addition, several outcome frameworks widely used in mammalian vaccinology are still underutilized in aquaculture. These include transmission-based endpoints [64, 65], time-to-event analyses [66], hazard-based modeling [67], and functional immune profiling beyond simple antibody titers [68, 69]. Together, these approaches provide a more detailed understanding of vaccine effects, allowing researchers to distinguish whether a vaccine reduces susceptibility, limits transmission, or primarily attenuates disease severity; hence, differences that are often obscured when relying solely on survival-based metrics in fish. Moreover, relying solely on survival-based endpoints can mask important aspects such as shedding rate, transmission potential, or subclinical infection, all of which have critical implications for population-level efficacy, pathogen circulation, and long-term disease control [70]. These limitations reflect a broader issue. Common outcome measures differ in the level of mechanistic insight they provide. There is a mismatch between what is easy to measure and what is biologically most informative. As a result, aquaculture vaccinology relies mainly on endpoints that show whether fish survive. However, these measures provide little insight into how, why, or under which real-world conditions protection occurs.

2.3 | When Protection Is Not Enough: Consequences of Suboptimal Vaccination

The consequences of partial (leaky) vaccine protection extend beyond individual fish capable of contributing to transmission either through detectable shedding or inferred persistence under cohabitation or field conditions. Suboptimal immunity can allow pathogens to circulate in vaccinated populations, producing complex and sometimes counterintuitive outcomes for pathogen circulation and evolution [71–75]. A clear example is infectious hematopoietic necrosis virus (IHNV) vaccination in rainbow trout (*Oncorhynchus mykiss*), where reduced mortality did not correspond with decreased viral shedding, allowing ongoing infection in the population [76, 77]. However, these outcomes should be seen as context-dependent rather than universal, since the level of leaky protection varies with pathogen biology, vaccine type, and transmission route [77, 78]. One possible explanation for this pattern is incomplete protection at mucosal surfaces. Although injectable vaccines can induce immune responses in mucosal tissues, potentially through migration of activated lymphocytes and local antibody production, the extent to which these responses provide durable protection at barrier sites remains incompletely understood. Because many

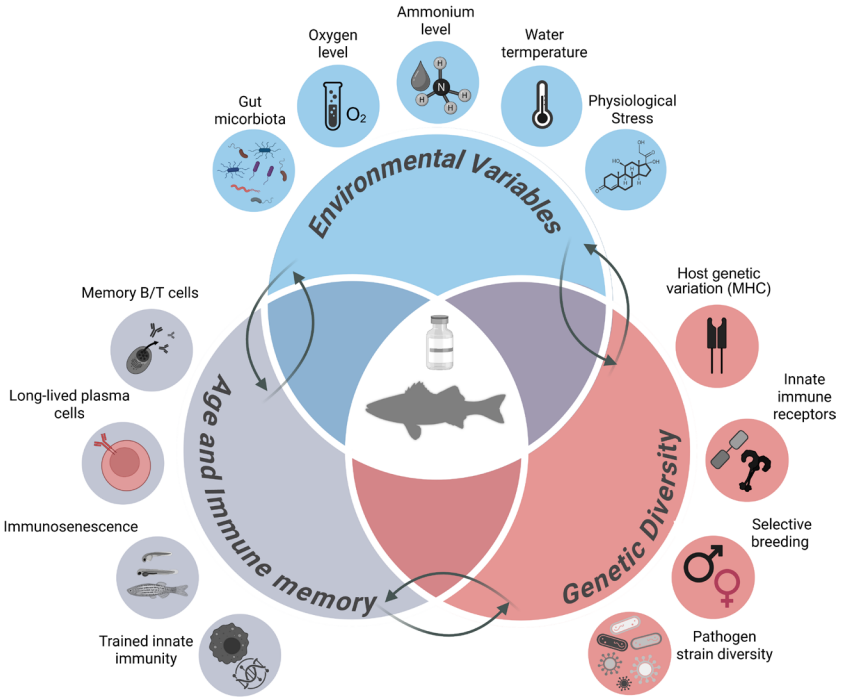

FIGURE 3 | Multi-dimensional determinants of vaccine efficacy in aquaculture. The efficacy of fish vaccines is governed by a complex interplay between host physiology, genetic background, and pathogen variability. MHC, major histocompatibility complex.

aquatic pathogens establish infection through the skin, gills, or gastrointestinal tract, vaccinated fish may sometimes survive disease while continuing to harbor and shed pathogens [79]. More broadly, the consequences of partial protection depend on the biology of the disease, how it is transmitted, and the epidemiological setting [80]. These factors together determine whether vaccination mainly reduces mortality, lowers infection rates, or limits onward transmission. Leaky vaccine outcomes have been observed for salmonid alphavirus 2 (SAV2) and infectious pancreatic necrosis virus (IPNV), where vaccination reduced pathology but did not consistently prevent infection, persistence, or shedding under cohabitation or field conditions [81–83]. Similar modulation of vaccine effects by co-infection has also been demonstrated for salmonid alphavirus subtype 3 (SAV3), where concurrent *Tenacibaculum dicentrarchi* infection altered shedding outcomes and reduced overall vaccine effectiveness [84]. Bacterial pathogens provide parallel examples. In infections caused by *P. salmonis*, vaccinated or partially immune fish may carry lower bacterial loads and shed fewer bacteria, yet remain infected and capable of contributing to transmission, particularly under stressful environmental conditions [85]. A comparable pattern has long been recognized for furunculosis (*A. salmonicida*), where vaccination effectively reduces disease outbreaks but does not consistently eliminate carrier states, enabling silent maintenance of the pathogen within fish populations [86]. Together, these cases illustrate how vaccination can decouple individual protection from population-level control. At broader scales, this divergence means that vaccination or genetic resistance may reshape disease dynamics without fully interrupting transmission. Such shifts can lead to complex and sometimes paradoxical outcomes for pathogen circulation and evolution [87]. Analogous phenomena are well documented in tetrapods, including Marek's disease in poultry and feline leukemia virus (FeLV) in cats [72, 88, 89]. These observations highlight the dangers of a "Black Box" approach, in which vaccines are deployed without understanding how host, pathogen, and environmental factors interact to determine efficacy. Conversely, partially protective vaccines can sometimes reduce overall pathogen load [90], illustrating the nuanced outcomes possible in complex ecological systems. Accordingly, some of the vaccines studies call for further investigation of this phenomenon [77]. Such findings underscore the need for detailed mechanistic studies to design vaccines that deliver consistent, durable, and evolutionarily sustainable protection.

3 | External Forces Shaping Vaccine Outcomes

Field performance of fish vaccines is shaped by a complex interplay of environmental conditions and host characteristics (age, stress status, genetic background) (Figure 3). These factors contribute to the vaccine pitfalls in aquatic vaccinology. The same vaccine can produce strong protection under one set of conditions and fail entirely under another. A key gap in understanding how environmental conditions influence vaccine efficacy is the limited integration of mucosal immunity, which forms the primary interface between fish and their aquatic environment. In teleosts, the mucosal surfaces of the gills, skin, and gastrointestinal tract function as integrated barrier–immune–microbiome systems. These surfaces are continuously exposed to environmental fluctuations and play a central role in shaping host susceptibility and resistance. Environmental stressors such as temperature changes, salinity shifts, hypoxia, and poor water quality can therefore influence vaccine responses not only through systemic pathways but also by directly affecting mucosal barrier integrity and local immune regulation [91], thereby shaping the immunological context in which vaccination outcomes are determined [92]. Laboratory experiments typically

cannot reproduce the fluctuating water temperature, dissolved oxygen, ammonia levels, or pathogen diversity present in production ponds. Similarly, co-infections, local pathogen strains, and exposure doses rarely match the clean, standardized models used in laboratory studies. Mitigating these effects requires iterative collaboration between researchers and farmers, through optimization of vaccination timing, delivery methods, and farm management practices. Because environmental stressors can affect both mucosal barrier function and immune competence, they should be considered a central determinant of vaccine performance alongside mucosal and systemic immunomodulation. Together, these factors show that vaccine efficacy in fish emerges from a dynamic interaction between environmental conditions and host biology. Resolving this dimension of the "Black Box" will require understanding how fluctuating field conditions shape vaccines across real production systems.

3.1 | Environment as an Immunological Filter

Environmental conditions do more than provide a backdrop for the fish immune system; they actively shape it. In fish, being poikilothermic means that water temperature controls the pace of both innate and adaptive immunity [93–95]. Temperature is a widely studied environmental cue in fish, not only in reproduction and behavior but also in immune response and the progression of infectious diseases [96, 97]. Even small changes in temperature can alter the balance between host defenses and pathogen dynamics [98], causing vaccines to perform well under some conditions but fail under others [99]. This raises concerns about whether fish hosts remain sufficiently immunocompetent under ongoing climate change [100], potentially complicating the "Black Box" of vaccine efficacy, where even the most well-designed vaccines may fail to provide consistent protection in shifting environmental conditions. Eurythermal species provide clear evidence of such mucosal plasticity, as even moderate shifts within the physiological range induce structural changes in mucosal tissues. For instance, Crucian carp (*Carassius carassius*) and goldfish (*Carassius auratus*) exhibit hypertrophy of interlamellar cells following prolonged exposure to low temperatures, while Fathead minnow (*Pimephales promelas*) shows alterations in mucus cell morphology under cold acclimation [101, 102]. Such temperature-driven remodeling of mucosal barriers is directly relevant for immune competence and may contribute to variability in vaccine responsiveness under different thermal regimes. This highlights that environmental stressors do more than just slow metabolism. They induce a state of mucosal plasticity. These structural and microbial disruptions, such as altered mucus composition and reduced antigen accessibility, impair mucosal immune function, contributing to context-dependent efficacy of oral or immersion vaccines where formulations succeed at optimal temperatures but fail under thermal stress [103–105]. The following examples illustrate how thermal conditions steadily influence the efficacy of vaccines across species, pathogens, and vaccine technologies. Some thermal constraints have been confirmed in Atlantic salmon, where even modest deviations from the optimal window during the first days of post-vaccination sharply reduced the efficacy of the licensed *P. salmonis* vaccine [106]. In red hybrid tilapia (*Oreochromis* sp.), an attenuated immersion vaccine against *Francisella noatunensis* provided protection only when fish were immunized at 30°C, whereas cooling to 25°C or introducing temperature fluctuations eliminated protection entirely, revealing a tight temperature–size dependency [103]. Nile tilapias show the same sensitivity, with vaccine against *Streptococcus agalactiae* being markedly less effective at 21°C due to suppressed innate and adaptive immunity, whereas optimal responses occur at 25°C–29°C [107]. Temperature also defines vaccine success in other fish species. A formalin-inactivated viral hemorrhagic septicemia virus (VHSV) vaccine protected Japanese flounder only at their physiological optimum of 20°C, failing at both 12°C and 28°C [108]. In ayu (*Plecoglossus altivelis*), temperatures around 22°C induce metabolic stress and thymus shrinkage, weakening baseline immunity and diminishing responses to bacterial cold water disease vaccination [109]. Formalin-inactivated vaccine against *Tenacibaculum maritimum* similarly elicits stronger leukocyte recruitment and pathogen-specific IgM responses at higher temperatures [110]. Likewise, while DNA vaccination against VHSV can protect rainbow trout across 5°C–15°C, low temperatures favor innate mechanisms and delay adaptive immunity, while warmer temperatures accelerate antibody production and pathogen clearance [111]. In olive flounder (*Paralichthys olivaceus*), vaccine against VHSV generates faster and stronger protection at higher temperatures, though full immunity is still achievable at colder temperatures if sufficient degree-days accumulate [105]. The licensed vaccine against salmonid rickettsial septicemia (SRS) used in Atlantic salmon also depends on early thermal support to establish long-term protection [106]. Crucially, thermal effects extend beyond systemic immunity to mucosal effector layers, where exposure to elevated temperatures increases lysozyme activity, hepcidin expression, and IgM levels in skin mucus, while cold acclimation suppresses antigen presentation and immunoglobulin pathways but enhances early antiviral, macrophage, and mucus-associated responses in fish [112]. These data collectively demonstrate that thermal conditions dictate not only the magnitude but also the kinetics and mechanism of vaccine-induced immunity across species. At the same time, vaccine technologies differ in their temperature sensitivity, offering important lessons for future design. A single-cycle VHSV vaccine in olive flounder can induce protection even at low temperatures, overcoming the typical constraints on adaptive responses [105], and a bivalent vaccine against *A. salmonicida* can still induce antibodies and enhance innate immunity in Arctic charr (*Salvelinus alpinus*) adapted to cold environments [113]. Conversely, high temperatures can introduce new challenges. Oil-adjuvanted vaccines in Atlantic salmon produce more abdominal lesions, vertebral deformities, and growth impacts as temperatures rise, revealing the thermal dependence of side effects as well as efficacy [114]. Even live vaccines depend on thermal suitability, as shown in goldfish vaccinated against herpesviral hematopoietic necrosis, where protection was strongest at 25°C and driven primarily by cell-mediated immunity [115]. For ectoparasites, the thermal limitation is especially clear. The channel catfish (*Ictalurus punctatus*) vaccinated against *Ichthyophthirius multifiliis* at 15°C fails to mount antibody responses and experience high mortality, whereas warmer conditions allow robust immunity and protection [116]. Ultimately, these studies illustrate that temperature is not simply a contextual parameter but a central determinant of vaccine outcome, possibly dictating the speed, magnitude, and even the mechanism of protection. As the negative impacts of global warming on aquaculture production continue to grow [117], understanding and managing these temperature-dependent immune constraints become essential. Without integrating environmental physiology into vaccine design and deployment, the success of even the best-engineered

vaccines may remain vulnerable to the shifting thermal realities of modern aquaculture. Alongside temperature, other environmental factors such as dissolved oxygen and ammonia levels can significantly stress fish and modulate immunity, making it essential to define conditions that maximize vaccine efficacy under suboptimal farm settings. Low dissolved oxygen is one of the most critical environmental stressors in aquaculture and has profound effects on mucosal barrier integrity and immune responsiveness in fish. Hypoxic conditions induce extensive remodeling of mucosal tissues, particularly in the gills, where structural adaptations such as increased lamellar surface area and proliferation of interlamellar cell mass have been described in hypoxia-tolerant cyprinids including Crucian carp and goldfish [118, 119]. While these changes improve oxygen uptake, they may also alter mucosal permeability and local immune homeostasis. Similar responses have been observed in scaleless common carp (*Cyprinus carpio*), where hypoxia rapidly induced gill remodeling and modifications of mitochondria-rich cells associated with ion transport and mucosal defense [120]. Gastrointestinal mucosa is also highly sensitive to oxygen fluctuations. In Atlantic salmon, chronic exposure to hypoxia caused intestinal inflammation accompanied by temperature-dependent modulation of immune gene expression, including altered levels of pro- and anti-inflammatory cytokines [121]. These findings indicate that hypoxia not only compromises mucosal structure but also dysregulates immune signaling pathways critical for maintaining barrier function and pathogen resistance. This dysregulation is partly driven by a chemical breakdown of the mucosal barrier. Environmental fluctuations downregulate the expression of key mucin genes, which are essential for anchoring the resident microbiota [122]. When this chemical scaffolding is weakened, the resulting shift in microbial composition feeds back into the immune system, further aggravating local inflammation and undermining the stability required for vaccine-induced priming. A recent study also indicates that reuse water, characterized by low dissolved oxygen and other production-related stressors, can undermine vaccination efficacy, leading to higher susceptibility, coinfection, and mortality in farmed fish [123]. Chronic moderate hypoxia in Nile tilapia suppresses and delays antibody production, reduces lymphocyte counts and serum bactericidal activity, and thereby impairs the efficacy of formalin-inactivated *Vibrio anguillarum* vaccination [124]. Similarly, intermittent hypoxia at 30°C or 35°C diminishes immune gene expression, antioxidant enzyme activity, phagocyte function, and bacterial clearance, leading to impaired vaccine-induced protection and higher mortality [125]. Mucosal immune responses are particularly sensitive to environmental conditions. In rainbow trout, intestinal immune activation following vaccination is prominent in freshwater but largely dampened in seawater, illustrating tissue- and environment-specific modulation of stress and immunity interactions [126]. Moreover, the role of the microbiome in vaccine responsiveness is only beginning to be explored, with studies in channel catfish and common carp indicating that probiotics or variations in rearing water microbiota can influence vaccine-induced protection [127–132]. Interpreting these effects remains challenging, as seen in Atlantic salmon, where chronic hypoxia reduced immune responses to a *Vibrio* water-based vaccine, yet the control group was already exposed to moderately low oxygen, complicating assessment [133–135]. Ammonia accumulation from metabolism, feed waste, or high stocking densities represents another major environmental stressor. Sublethal ammonia exposure damages tissues, induces oxidative stress, impairs respiration, and compromises immune function, potentially reducing vaccine efficacy [136–139]. In rainbow trout vaccinated against *Streptococcus iniae*, low to medium ammonia levels had minimal effect, whereas high or prolonged exposure significantly lowered relative percent survival (RPS). Notably, antibody titers did not correlate with protection, suggesting that ammonia impairs cellular or nonspecific immune mechanisms, or affects other physiological systems such as vascular or respiratory function [140].

3.2 | Stress: The Silent Saboteur of Vaccine Efficacy

Vaccination itself can act as a physiological stressor in teleost fish, often provoking marked elevations in circulating cortisol [141–143] and glucose [144]. This stress response is particularly pronounced following IP injection, which consistently induces higher cortisol and hyperglycemic peaks compared with immersion-based or oral immunization, highlighting the influence of delivery route on physiological burden [145, 146]. Consequently, fish receiving only mucosal priming typically exhibit a far milder endocrine disturbance than those subjected to an IP boost [145]. Such vaccine-associated stress may not be a trivial side effect. The elevated cortisol can modulate immune function, impair growth, and ultimately reduce disease resistance [146]. Moreover, stress can interfere with antigen processing and presentation, potentially diminishing vaccine efficacy at both systemic and mucosal sites. Another vaccine-oriented study found that routine vaccination and associated handling in Atlantic salmon pre-smolts induced acute stress and distinct immune gene responses [147]. These shifts help explain the increased susceptibility to saprolegniosis observed after vaccination on farms [147]. Short-term stress and vaccination elicit species-, tissue-, and time-specific immune and stress responses in fish, with the liver generally showing greater transcriptional responsiveness than the spleen [148]. For example, bath vaccination with *V. anguillarum* bacterin and handling stress induced differential cortisol and gene expression patterns across seabream, zebrafish (*Danio rerio*), and trout, highlighting that stress–immune interactions and vaccine responsiveness are highly dependent on both species and the type of stressor [148]. At the mucosal level, stress-induced cortisol release exerts a multitiered impact on the mucosal barrier [149]. While vaccination-induced stress primarily manifests through cortisol elevation, the resulting impact on the microbiota is a complex, multi-way interaction influenced heavily by the aquatic environment [150, 151]. Stress-induced immunosuppression is thought to allow resident commensal bacteria to overgrow, which can lead to a shift from a symbiotic state to one of dysbiosis. Physically, it compromises epithelial integrity by inducing a widening of tight junctions [152, 153]. Chemically, stress alters the mucosal shield by downregulating mucin gene expression [154], while biologically, it triggers dysbiosis, a shift in the microbiota that reduces microbial diversity and eliminates beneficial populations that provide competitive exclusion against pathogens [155–159]. Furthermore, cortisol modulates the activity of resident immune cells, including IgT^+ B cells, and suppresses the expression of key pro-inflammatory cytokines in the gut-associated lymphoid tissue [121, 135]. These structural and microbial disruptions weaken the capacity of mucosal tissues to mount effective responses to vaccination, particularly for delivery strategies that rely on local immune activation. Because

the aquatic environment highly supports microbial growth, even minute changes in the immune status of the host can trigger rapid states of dysbiosis. This transition to dysbiosis is often defined by microbial concentration. For example, the skin commensal *Staphylococcus warneri* promotes anti-inflammatory health at low levels but triggers pro-inflammatory responses if allowed to overgrow during periods of stress [160]. This highlights a delicate triangle of interaction between the host, the microbiota, and the surrounding water [156]. Beyond the reaction of the host, stress hormones can be directly sensed by commensal microbes, altering their behavior and growth. Practical examples underscore this sensitivity. For instance, transportation stress has been shown to significantly increase the total number of culturable bacteria in skin mucus [161]. Similarly, hypoxic stress can shift the microbial balance to favor opportunistic pathogens from *Aeromonas* and *Pseudomonas* genera [156]. Despite these disruptions, the teleost microbiota possesses internal stability mechanisms, such as functionally redundant members that become more abundant to preserve community function during perturbations. Furthermore, protected microenvironments may serve as essential reservoirs for the recolonization of mucosal surfaces once the stressor is removed [150, 162]. Recent studies further demonstrate that vaccination in rainbow trout triggers a systematic level-based long-lasting stress response, particularly in the gills, as evidenced by upregulation of neuroendocrine, cellular, oxidative, and inflammatory genes [163, 164]. This indicates that mitigating vaccination-induced stress is essential to optimize vaccine effectiveness at mucosal surfaces [163]. Importantly, novel approaches are emerging to reduce vaccination-induced stress. Plant-based sedatives such as essential oil treatments have been shown to mitigate both physiological and molecular stress responses in sea bass following IP vaccination [165]. Alternatively, emerging psychobiotics (psychoactive probiotics) may contribute to stress mitigation [166]. However, only a limited number of such compounds are currently available, and the effects of the gut microbiota are unknown. Such complementing chemical interventions, minimizing handling stress prior to vaccination can thus further reduce negative physiological effects, improving both animal welfare and vaccine performance [167]. Collectively, these findings underscore that the method of vaccine delivery, species-specific stress responses, and farm management practices all interact to shape the efficacy of immunization programs. Addressing stress through optimized delivery routes, pre-vaccination sedation, and careful handling represents a practical strategy to enhance both immune protection and welfare outcomes in aquaculture.

3.3 | Genetic Diversity: Why One Vaccine May Not Fit All

Among all the intrinsic factors shaping vaccine performance in fish, host genetics stands out. It represents a major component of the "Black Box" governing vaccine efficacy, where even small differences in genotype can fundamentally alter how a fish recognizes a pathogen and ultimately whether vaccination provides protection or not [51]. For instance, subfamilies of Atlantic salmon differ markedly in protection conferred by *P. salmonis* vaccines, with some families gaining substantial survival, while others derive little or even no benefit [53]. A genome-wide association study further supports this complexity, emphasizing polygenic resistance to *P. salmonis*, with multiple loci of small effect contributing to variation among individuals [168] and a recent study also deepens this issue to the level of networks and genes mediating such genetic resistance [169]. Genetic polymorphisms in immune genes, especially the Major Histocompatibility Complex (MHC), likely underpin this host-level variation. Strong associations have been reported between MHC class I and II alleles and disease resistance in salmon challenged with *A. salmonicida* or infectious salmon anemia virus (ISAV) [170], while clinal variation in MHC class II diversity across populations correlates with local pathogen richness [171]. Pathogen-strain diversity interacts with host genotypes to further modulate vaccine effectiveness. For example, commercial vaccines against *P. salmonis* often fail against widely circulating genogroups, highlighting that a vaccine derived from one strain may not work equally well across genetically and antigenically diverse host populations [7]. Such use of non-local pathogen strains in vaccine development has been frequently reported as the result of reduced efficacy when deployed in diverse geographic contexts [53, 172–174]. Paradoxically, reported outcomes continue to diverge, with vaccines proving effective in some cases but failing in others in terms of cross protection of vaccine for different strains of pathogen [54, 175]. Gene expression studies in vaccinated salmon reveal distinct immune profiles when challenged with different *P. salmonis* isolates, including variation in MHC class I/II, CD4, and IgM expression [176]. This demonstrates how antigen mismatch can break the code, contributing externally to the "Black Box" problem where observed protection is inconsistent and mechanistically opaque, although in this case the extent of this effect is still to be evaluated. To overcome these challenges, selective breeding for vaccine responsiveness, polyvalent or strain-matched vaccines, and precision vaccination strategies tailored to population or family-level genotypes may be required. It is possible that without integrating host genetic diversity into vaccine design and deployment, persistent gaps in protection will undermine vaccine efficacy and compromise disease control in aquaculture, although massive genotyping and genetic matching of vaccines is a song of the future.

3.4 | Age Matters: Developmental Window That Define Vaccine Success

In fish, the age at vaccination sets the stage for success or failure. The readiness of the immune system at the moment of exposure often dictates whether protection takes hold or fizzles out. This relationship stems from the ontogeny of the immune system [177], the presence of maternally derived antibodies [178, 179], and the capacity to mount adaptive responses. Consequently, optimizing vaccination timing is crucial to maximize protective efficacy. Because adaptive immunity is not fully mature in very young fish, vaccination at too early a stage often fails to confer long-term protection. For instance, in Gilthead seabream (*Sparus aurata*), although gene markers for lymphocytes appear between approximately 21 to 48 days post-hatching, immersion or oral vaccination in young larvae increased susceptibility to infection, suggesting an immature or dysregulated adaptive response [180]. In contrast, Asian seabass (*Lates calcarifer*) fry of a similar age could mount a specific immune response after immersion vaccination, indicating that some functional immunocompetence emerges relatively early in certain species [181]. Similarly, in Nile tilapia, immersion vaccination with

formalin-killed *Streptococcus agalactiae* elicited detectable specific IgM from 21 days after yolk sac collapse, with stronger and faster responses in older larvae, conferring significant protection at all stages tested [182]. In salmonid species, however, protective immunity against *V. anguillarum* or *Y. ruckeri* can only be achieved after a certain fry age, highlighting a minimum threshold of immune maturity required for effective vaccination [183]. This age- or size-dependent effect is also observed in other species. In Japanese flounder, protective immunity against viral hemorrhagic septicemia virus develops only in juveniles above a certain size, as smaller fish lack effective antiviral responses and virus-specific antibodies [184]. In Japanese amberjack (*Seriola quinqueradiata*), vaccine-specific IgM and protective immunity against *V. anguillarum* appear only after approximately 60 days post-hatching, reflecting a delayed functional humoral response relative to initial IgM production [185]. Likewise, in Ballan wrasse (*Labrus bergylta*), immersion vaccination against atypical *A. salmonicida* is ineffective in juveniles <1.5 g, consistent with the late onset of adaptive immune responses, as indicated by the first expression of MHC class II and IgM transcripts at 35- and 75-days post-hatch, respectively [186]. Taken together, these studies highlight that the timing of vaccination in fish is constrained by species-specific windows of immunocompetence. Collectively, these studies indicate that age-dependent vaccine efficacy in fish reflects the attainment of a functional immunological threshold rather than chronological age per se. Early life stages are characterized by incomplete lymphoid organ maturation, limited antigen-presenting capacity, restricted lymphocyte repertoire diversity, and weak coordination between innate and adaptive immune signaling, all of which constrain the ability to translate antigen exposure into durable protection [178, 187]. Although transcription of immune markers such as IgM or MHC molecules may occur relatively early, protective immunity emerges only once immune architecture and cellular interactions have reached sufficient maturity to support effective priming, clonal expansion, and memory formation. From this perspective, age serves as a proxy for immune system readiness, defining a developmental window in which vaccination can be productively integrated into long-term host defense rather than eliciting transient or maladaptive responses. Understanding these developmental thresholds is critical for designing effective vaccination strategies in aquaculture.

4 | Segment of the Black Box: Immunological Mechanisms That Decide Vaccine Fate

Despite decades of progress in aquaculture vaccinology, persistent bottlenecks often arise not from antigen discovery or formulation, but from an incomplete understanding of fish-specific immunology. With over 30,000 recognized fish species, each adapted to its ecological niche, there is no universal fish immune system but only thousands of variations shaped by evolution, anatomy, and environment [188]. These differences fundamentally influence how antigens are processed [189], how immunity is distributed across systemic and mucosal tissues, and how durable protection can realistically be (Figure 4). Understanding these immunological features is essential not just for selecting antigens and adjuvants, but for interpreting why vaccines that appear highly immunogenic in laboratory trials may underperform in the field. As immersion, oral, and mucosal vaccines become more common trends, effective design increasingly

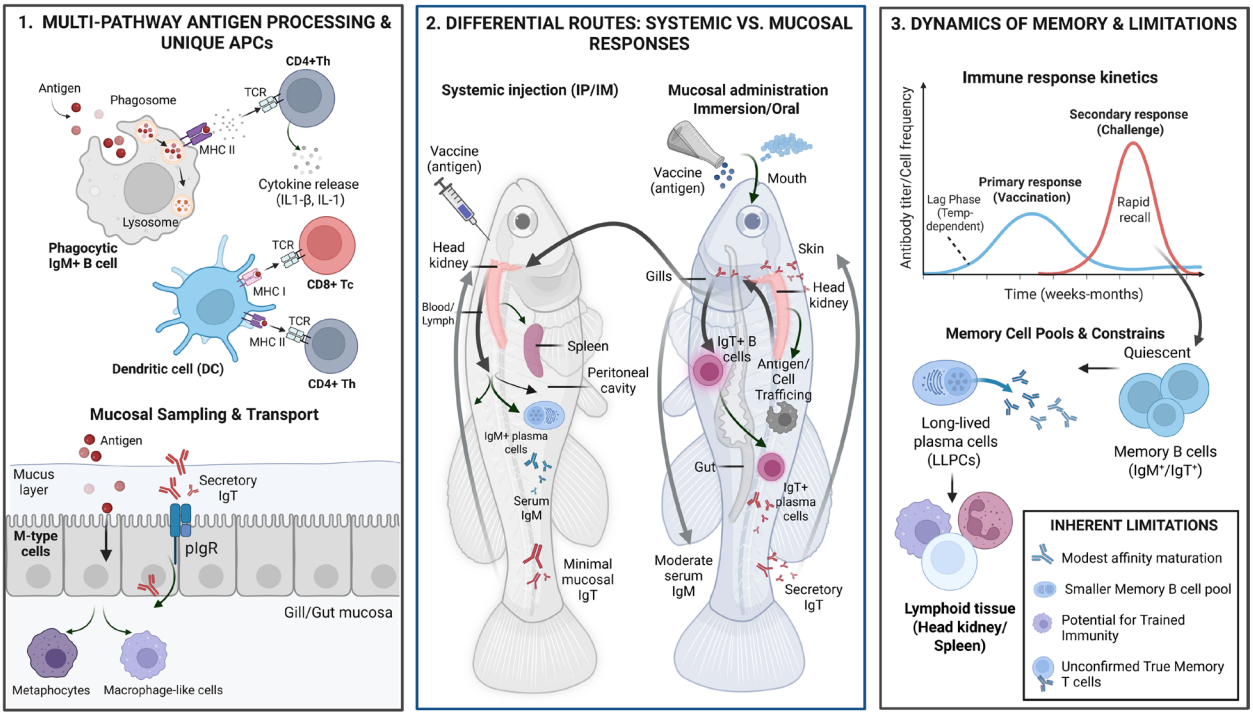

FIGURE 4 | Schematic illustration of key immunological processes following vaccination in teleost fish. Panel 1 depicts antigen processing by unique antigen presenting cells (APCs) and mucosal sampling cells. Panel 2 displays the distinct immune outcomes of systemic versus mucosal vaccine delivery. Panel 3 details the dynamics of immune memory, including response kinetics and the inherent limitations on long-term protection in fish. DC: dendritic cell; IM: intramuscular injection; IP: intraperitoneal injection; pIgR: polymeric immunoglobulin receptor; Tc: cytotoxic T cell; TCR: T cell receptor; Th: T helper cell.

depends on a mechanistic grasp of teleost immunity, moving beyond empirical formulations toward vaccines engineered to work with the host immune system rather than against it.

4.1 | Antigen Processing and Presentation: Hidden Gatekeepers of Immunity

Antigen processing and presentation (APP) is the gateway to immune activation in teleost fish, and the route of vaccine delivery often dictates the outcome. Systemic versus mucosal entry engages distinct antigen-presenting cell (APC) populations and MHC pathways. Most teleosts possess canonical MHC class I and II machinery but deploy it through an architecture that diverges from mammals: mucosal tissues like gills, gut, and skin act as frontline sites rich in IgT/IgZ responses, polymeric immunoglobulin receptor-mediated transport, and unconventional APCs [157, 190]. Professional APCs like dendritic cells (DCs), macrophages, and B cells exhibit tissue-specific functional specialization. In zebrafish, myeloid cells analogous to mammalian DCs phagocytose bacteria and stimulate T-cell proliferation [191]. Rainbow trout possess DC-like subsets in skin and gills capable of cross-presentation, while intestinal DCs show unique phenotypes supporting mucosal immunity [192, 193]. Given additional players, teleost B cells are not just antibody factories, but also phagocytes and APCs. Teleost IgM^+ B cells ingest particulate antigens, express pattern recognition receptors (PRRs), and process antigen via MHC class II to activate $CD4^+$ T cells [194, 195]. In zebrafish, these B cells upregulate costimulatory molecules (CD80/86, CD83) during activation, like dendritic cells, positioning them as initiating APCs [195]. Studies in grass carp (*Ctenopharyngodon idella*) show that IgM^+ plasma-like cells can phagocytose and secrete antigen-specific IgM, indicating an evolutionary intersection between B-1 and B-2 cell functionality [196]. In tilapia, a subset of IgM^+ cells exhibit plasma-cell morphology, high secretory capacity, robust phagocytosis, and antigen processing [197]. In mucosal tissues, specialized antigen-sampling cells amplify the diversity of APC functions. In rainbow trout gill epithelium, M-type cells have been discovered that constitutively express MHC class II and significantly upregulate it following antigen uptake [198]. These M-type cells also possess gene expression signatures for phagosome, lysosome, and antigen presentation pathways, suggesting they contribute directly to antigen presentation. Furthermore, metaphocytes, ectoderm-derived, non-phagocytic myeloid-like cells, in zebrafish skin, gill, and gut take up soluble antigens and shuttle them to other immune cells, thus acting as antigen transporters rather than classical phagocytes [199]. The efficiency of this cellular interaction determines whether a mucosal vaccine translates into protective immunity or transient exposure. Unlike systemic injection, which bypasses these frontline samplers, immersion and oral vaccines rely on this specialized shuttling network to initiate local responses. The performance of oral vaccines is fundamentally linked to the site of antigen deposition [13]. Evidence shows that the second segment of the gut is the primary site for antigen-presenting cells (APCs) [200, 201]. Studies using anal intubation of *V. anguillarum* vaccines demonstrate that targeting this specific segment, rather than the first segment, results in significantly higher antibody levels in the skin mucus and bile [202]. This suggests that mucosal division of immune labor allows for a localized stimulus to generate a distal protective response across different mucosal surfaces [202]. This reliance on a highly tissue-specific cellular infrastructure provides a direct mechanistic explanation for the performance variability often observed in non-injection delivery routes. The interspecies variation in APC and MHC architecture can dramatically influence how antigen is handled. For example, Atlantic cod (*Gadus morhua*) lacks classical MHC class II, CD4, and invariant chain genes, but compensates with a dramatic expansion of MHC class I loci, suggesting reliance on cytosolic pathways [144]. The absence of MHC class II in cod has been discussed in evolutionary terms, with hypotheses ranging from metabolic cost reduction to functional compensation [203]. Recent work has also shown that cod can mount strong T cell-independent antibody responses, reinforcing the idea that its immune architecture deviates from classical paradigms [189]. From a vaccinology perspective, delivery format dictates, which APC pathways are engaged and thereby shapes the immune outcome. Intracellular delivery (e.g., DNA vaccines, live attenuated) favors endogenous antigen processing and presentation on MHC class I, driving cytotoxic T-cell responses [204]. In contrast, extracellular antigen formulations (e.g., inactivated pathogens, subunit vaccines) must be internalized by phagocytic APCs (macrophages, DCs, phagocytic B cells, M-type cells) and presented via MHC class II, eliciting helper T-cell and antibody responses [205]. The size, structure, and stability of antigen (e.g., particulate vs. soluble, polymeric, opsonized) further influence which APC subsets pick them up (e.g., B cells vs. DCs) and how efficiently presentation occurs. In short, APC biology in teleost fish is an active, delivery-sensitive network, and how antigens encounter the host largely decides vaccine success.

4.2 | Systemic Versus Mucosal Immunity: Vaccine Stimulation

Most fish vaccines induce strong systemic IgM responses measurable in serum, but many pathogens first interact with mucosal surfaces, where IgT and local cellular responses are considered dominant [91, 190, 206]. Consequently, systemic antibody titers often overestimate actual protection at these portals of entry. Unless a vaccine can stimulate a local mucosal response or facilitate effective trafficking of systemic effectors to the mucosa, protection against natural exposure may remain incomplete [12, 14]. The route of antigen delivery strongly influences the balance between systemic and mucosal immune responses. Mucosal administration (oral, anal, immersion) preferentially stimulates local immune compartments, whereas systemic delivery (intraperitoneal or intramuscular injection) generally induces higher serum antibody levels [12]. For antibacterial vaccines, studies in multiple teleost fish models show that intraperitoneal injection typically elicits the highest systemic IgM titers [202, 207–210]. In contrast, anal intubation, oral, or immersion vaccination enhances mucosal antibody production, particularly in skin, gill, and gut mucus [202, 207, 210]. Notably, mucosal vaccines can induce significant local responses even when systemic antibody titers remain low, as seen with *Y. ruckeri* or *Flavobacterium psychrophilum* immunizations in rainbow trout [210, 211], consistent with local priming of mucosal B-cell responses at pathogen entry sites. Secretory IgT and IgD antibodies are predominantly elevated in mucosal tissues following immersion or anal immunization [210], highlighting tissue-specific immune activation.

Similar patterns are observed for antiviral vaccines. Oral, immersion, or encapsulated formulations of VHSV and nervous necrosis virus (NNV) increase both mucosal and systemic antibody titers and induce transcription of IgM, IgT, polymeric immunoglobulin receptor (pIgR), and MHC class I/II in mucosal and systemic organs [212–215], suggesting coordinated activation of barrier and systemic antiviral immunity. These studies demonstrate that mucosal vaccination can elicit coordinated local and systemic responses, although systemic routes generally maximize circulating antibody levels. Recent studies further illustrate the benefits of mucosal-targeted vaccination. In red tilapia, immersion delivery of a mucoadhesive nanovaccine elicited strong IgT and IgM expression in both mucosal tissues and systemic organs, conferring protection against *F. columnare* [216], likely through prolonged antigen retention and enhanced activation of mucosal immune compartments. In rainbow trout fry, a whole-cell immersion vaccine against *F. psychrophilum* upregulated IgT in the gut while expanding systemic IgT^+ B cells, leading to high survival upon challenge [217], illustrating how mucosal stimulation can drive coordinated local and peripheral IgT responses. Oral vaccination of olive flounder with an *Edwardsiella tarda* vaccine enhanced intestinal antigen-specific antibody responses and improved survival [218], consistent with direct stimulation of gut-associated immune tissues. By contrast, feeding recombinant *Lactobacillus casei* expressing *Aeromonas veronii* antigen to common carp stimulated both mucosal and systemic IgM responses [219], highlighting the capacity of mucosal antigen delivery to bridge local and systemic immunity. These findings underscore strategies priming mucosal B and T cells that finally yield superior protection against pathogens entering through mucosal surfaces [220, 221]. Taken together, these studies demonstrate that vaccine efficacy is strongly shaped by the anatomical site of antigen encounter, with mucosal delivery preferentially engaging barrier-associated immune networks that are often poorly stimulated by systemic vaccination alone. Systemic–mucosal crosstalk therefore emerges as a central determinant of effective protection in teleost fish.

4.3 | Antibody Responses: Deconstruction of Vaccine Efficacy and Evaluation

Despite decades of progress, teleost humoral immunity is only partially understood, in which antigen exposure reliably induces measurable antibody responses, yet the molecular logic underlying antibody generation and protection remains poorly defined. B-cell activation can be broadly traced from antigen encounter to effector antibody production, yet the upstream decision rules governing whether responses become systemic or barrier-localized remain unresolved, limiting prediction of vaccine outcomes. B cells then undergo diversification and selection in the apparent absence of classical germinal centers, implying alternative affinity-maturation pathways that remain poorly characterized, complicating interpretation of antibody quality and durability. Importantly, antibody abundance does not consistently correlate with protection [5], underscoring a persistent disconnect between serology and functional immunity. The core effector system is built around a triad of immunoglobulin isotypes, IgM, IgT, and IgD, which originate from a common B-cell lineage but diverge in structure, localization, and function. Rather than a simple systemic versus mucosal division, these isotypes define partially specialized immune programs [222–227], whose regulation remains a major limitation for rational vaccine design and interpretation of compartment-specific vaccine responses. Following antigen exposure, immune signals differentially engage systemic IgM^+/IgD^+ B cells or mucosal IgT^+ B cells, establishing compartmentalized responses, although the mechanisms governing this site-specific specification remain poorly defined. IgT represents a mucosal-specialized axis, with IgT^+ B cells enriched in barrier tissues such as skin, gills, and gut, where they mediate local pathogen-specific responses distinct from systemic IgM activity [228]. However, the pathways controlling IgT induction and lineage commitment remain unknown. Although microbiota–immune interactions are conserved and bidirectional in teleosts [229, 230], how environmental cues shape IgT^+ B-cell development is still unresolved, limiting rational design of mucosal vaccines that reliably induce IgT-dominated immunity. IgD adds further complexity. It is evolutionarily conserved and typically co-expressed with IgM on naïve B cells via alternative splicing [231, 232]. In teleosts, IgD^+IgM^- B cells are enriched in mucosal tissues [233, 234] and can differentiate into IgD-secreting plasmablast-like cells [233]. Secreted IgD interacts with commensal microbiota and shows clonal expansion, suggesting a conserved role in mucosal homeostasis and host–microbiota mutualism [233].

At the architectural level, teleosts lack classical germinal centers but form inducible melanomacrophage center–associated lymphoid aggregates where B-cell expansion [235], limited somatic hypermutation [236], and selection occur. Infection-driven VH repertoire skewing further supports antigen-driven clonal selection [223]. However, the absence of canonical germinal center organization complicates interpretation of affinity maturation, memory formation, and vaccine durability. Uncertainty also remains regarding long-lived plasma cell–like states versus short-lived plasmablasts, despite evidence for persistent memory-like IgM^+ B cells [237]. This further confounds prediction of vaccine durability and booster requirements.

At the effector level, IgM is the dominant systemic antibody and primary serological correlate of vaccination. It forms J-chain–independent tetramers stabilized by Fc interactions, with structural diversity that likely enhances avidity, although the relative contribution of affinity versus avidity to protection remains unclear [238]. IgT is monomeric in serum but forms multimeric complexes in mucosal mucus, where it is enriched and specialized for barrier immunity [239]. IgD is primarily monomeric, with secreted forms also detected, indicating a distinct non-oligomeric architecture [239]. However, high-resolution structural data for these immunoglobulins remain limited, restricting mechanistic interpretation of function.

Antibody distribution further limits functional inference. Polymeric immunoglobulin receptor (pIgR)-mediated transcytosis is a major pathway for IgM and IgT transport to mucosal surfaces [206, 240–245], with emerging evidence suggesting IgD involvement as well [242], although class-specific transport mechanisms remain unresolved. Repertoire analyses show broad systemic IgM expansion but restricted IgT responses [223], reinforcing strong compartmentalization of humoral outputs and limiting the predictive value of circulating antibody levels.

Finally, antibody abundance does not reliably predict protection. While neutralizing activity is observed across viral systems [245], protection often depends on additional cellular and innate mechanisms. In rhabdovirus infections, public antibody responses converge on shared neutralizing clonotypes [246], whereas bacterial protection relies more on opsonization and complement activation [246]. Secretory IgM and IgT contribute to mucosal defense [222], challenging strict functional segregation. Nevertheless, the primordial principles and mechanisms of these antibodies in fish are still an active area of research [229, 230, 247–249]. Yet, complement pathways [250–252], Fc-mediated effector functions [222], and Fc receptor signaling remain poorly defined [253, 254], leaving major gaps in mechanistic understanding and limiting identification of functional correlates of antibody-mediated vaccine protection. Collectively, these findings indicate that effective vaccination in teleosts requires not only antibody induction, but precise control of antibody specificity, localization, and effector coordination across systemic and mucosal compartments.

4.4 | Cellular Immunity: A Parallel Driver of Vaccine Protection

Cell-mediated immunity in teleosts represents an evolutionarily conserved counterpart of mammalian adaptive immunity, yet its contribution to vaccine-induced protection remains largely inferred rather than directly demonstrated. $CD4^+$- and $CD8^+$-like T-cell lineages are well described in fish [14, 255, 256], and vaccine studies consistently report modulation of CD4/CD8-associated transcripts and cytokine signatures [257–261], but these remain predominantly transcriptional, leaving a persistent chasm between immune activation and confirmed effector function. This disconnect is rooted in unresolved T-cell biology in teleosts. Vaccine-induced IFNγ and IL-2–like expression in salmonids and common carp [262], suggests T-cell–linked activation supporting adaptive immunity, yet whether these signals map to discrete Th-like lineages or conserved transcriptional programs remains unclear. This ambiguity is intensified by the lack of functional perturbation tools such as depletion, adoptive transfer, or lineage tracing in fish, preventing direct assignment of function to T-cell subsets. Evidence nevertheless points to a cytotoxic axis in antiviral protection. In teleost vaccine systems, MHC-I and CD8-associated signatures consistently correlate with protection, including oral vaccination against nervous necrosis virus (NNV), where MHC-I and CD8α are upregulated in gut tissues [215]. This supports a functional role for MHC-I–restricted cytotoxic responses in mucosal immunity [263], although direct in vivo evidence of T-cell killing remains limited [264].

Given this framework, the central challenge is no longer description but definition of functional indicators of T-cell immunity. $CD4^+$ responses remain widely used as immune proxies [265–268], but their functional differentiation into Th-like subsets is still unresolved [269], despite evidence that T cells coordinate both cytotoxic activity and IgM^+ B-cell responses via CD3/CD28 signaling in Nile tilapia [270]. $CD8^+$ responses are more consistently linked to antiviral protection [264] and are enhanced by MHC-I–promoting vaccine platforms [264, 271]. Accordingly, CD8 and IFNγ transcripts represent early activation markers [272], while granzyme B-like activity provides a conserved effector readout across species [273]. Similar resolution can be reached at mucosal regulatory axes such as p53-dependent pathways that further link antigen uptake to coordinated T and B cell activation [274]. These responses are further shaped by spatial immune compartmentalization. Mucosal tissues exhibit rapid, CD8-biased responses, whereas head kidney and spleen support systemic T-cell cooperation [14, 275–281]. Mucosal vaccination induces coordinated CD4, CD8, MHC I, and MHC II expression [282–284] while CD8-dominant signatures in hindgut and pyloric caeca suggest primary effector activity at mucosal surfaces [213, 214, 277, 285–287]. In contrast, systemic vaccination induces broader systemic activation without mucosal residency [288–290], complicating interpretation of protective correlates.

Despite these advances, most readouts remain correlative. Resolving this requires perturbation-based validation such as cell depletion or adoptive transfer [291], alongside assessment of effector–memory dynamics and systems vaccinology approaches [292–294]. Complementary mechanistic markers, including LAT-signalosome and CD3/CD28 signaling components (S6 phosphorylation, NFAT1, CD122, CD38-mediated B-cell help [295] and TCR repertoire dynamics [296–299], may provide functional resolution beyond single-gene endpoints). These findings highlight a central unresolved issue in teleost vaccinology: cellular immunity is consistently detectable, but not yet functionally defined.

4.5 | Immune Memory and Durability: Why Protection Fades Over Time

In fish, as in all vertebrates, the immune system does not forget. Once exposed to a pathogen or vaccine, teleosts can remember the encounter, thanks to long-lived IgM^+ memory B cells and plasma cells that settle in lymphoid organs like the head kidney and spleen, continuously producing antibodies and ready to spring into action on re-exposure [91, 237]. Following a first encounter, there is a natural lag while B cells multiply and differentiate. A process that ticks faster in warm-water species than in cold-water fish [95, 300]. When the same pathogen appears again, the immune system responds like a well-prepared team. The antibody levels rise rapidly, often outpacing the primary response and pointing to T cells ready to deliver enhanced cytotoxic activity [301]. Despite functional evidence of secondary T cell responses, the presence of bona fide memory T cells in fish has not yet been conclusively demonstrated. In practical terms, a single vaccination in juvenile fish can carry protection through an entire grow-out period of 6–12 months, although boosters or memory-promoting adjuvants can help keep immunity at full strength [14, 237, 302]. Despite some species- and environment-dependent limits, the principle is clear: building robust B- and T-cell memory is at the heart of effective vaccination in aquaculture. Beyond classical adaptive memory mediated by B and T cells, recent studies highlight additional layers of immune memory in fish. Teleost fish exhibit trained innate immunity, in which macrophages and other myeloid cells display enhanced responsiveness upon re-exposure to pathogens, potentially complementing classical memory in vaccination [303–305]. However, the lack of well-defined surface markers for memory lymphocytes in

most teleost species limits our ability to track these populations directly, so memory is often inferred from recall responses or antibody kinetics [91, 237, 306]. Omics approaches are increasingly being applied to bypass these limitations by high-dimensional dissection of B- and T-cell heterogeneity [307–310], however, memory lymphocyte populations remain challenging to define across teleosts [308, 310, 311]. Notably, a comprehensive single-cell atlas in humans has demonstrated that conventional isotype-based gating strategies are insufficient to resolve the full functional and metabolic diversity of the B-cell compartment. Indeed, integration of mass cytometry, V(D)J repertoire sequencing, and metabolic profiling has revealed that discrete functional states are more accurately delineated by coordinated transcriptional and surface-marker signatures than by antibody isotype alone [312]. Despite these advances, robust transcriptional demarcation of memory lymphocytes remains problematic. Classical adoption of mammalian memory-cell classification criteria in teleosts may be misleading in both directions and cannot be considered definitive. In humans, CD27 has long been regarded as a canonical memory B-cell marker [313], although recent evidence questions its specificity [314, 315]. In teleosts, however, available data suggest a different functional context. For example, a recent study in Nile tilapia reported predominant CD27 gene expression in CD4+ T cells and suggested broader roles in inflammatory and immune signaling pathways rather than a specific association with memory B-cell identity [316]. This complicates retrospective interpretation of conserved lymphocyte states across the large evolutionary distance separating teleosts and mammals. Within this context, tissue residency and cellular origin can influence surface marker expression, thereby blurring the boundaries between memory B-cell subsets across distinct immune niches [313]. Furthermore, affinity maturation is modest in teleosts, and long-lived plasma and memory B-cell pools are smaller than in mammals, placing an upper limit on the magnitude and quality of vaccine-induced memory [130, 169]. Ultimately, bridging the gap between indirect inference and a mechanistic "White Box" of fish vaccine efficacy requires adopting the paradigm that protective immunity is a multifaceted architecture of layered, porous barriers [317]. As demonstrated in human vaccinology, durable protection relies on the integrated activity of antigen-independent, tissue-resident memory acting as a local control barrier at the site of entry and circulating memory providing a secondary systemic backup. Transitioning to such a systems-level characterization is essential to determine if teleosts possess these discrete functional layers or have evolved phenotypically distinct but functionally analogous strategies for long-term survival.

5 | Toward a "White Box" in Fish Vaccinology

In complex biological systems, progress does not begin with resolution but with perspective. Therefore, a "Box" analogy must first be understood as a coherent whole. In vaccine testing, "Black Box" evaluation focuses on outcomes, whereas a "White Box" approach fully reveals the processes that generate these outcomes. However, the transition from one to the other is not a simple linear shift based on the accumulation of research breakthroughs, but rather a coordinated interplay between research and innovation, translational implementation, and regulatory governance. This includes not only studying the problem itself, particularly how the complex immune system responds to vaccination and its downstream effects but also addressing the external factors and applied side in parallel. This ranges from precise laboratory and field protocols and procedures to the broader legislative framework. Such regulatory oversight is essential for monitoring, controlling, and evaluating vaccine efficacy, from initial assessment through to real-world deployment. To address the route from black to white within the "Box" concept in operational terms, the following roadmap is structured according to feasibility and implementation priority (Figure 5). The three tiers represent (i) immediate priorities that enable standardization and comparability across studies, (ii) medium-term methodological consolidation focused on mechanistic integration, and (iii) long-term developments aimed at predictive and regulatory transformation of vaccine evaluation systems.

5.1 | Immediate Priorities: Pathogen Characterization and Standardization

The entry point into this transition is the pathogen itself, the ultimate target of any vaccine strategy. Its biology defines the core constraints for vaccine design, including how it enters the host, which tissues it targets, how it spreads, how frequently exposure occurs, and whether it can induce immune memory. These are not abstract characteristics but defining parameters that determine the realistic limits of vaccine efficacy. In this sense, pathogen characterization represents the first step in structuring an otherwise complex and only partially defined system. From this foundation, the first level of resolution emerges through standardization. In the short term, the primary objective is to identify robust and reproducible correlates of protection. These markers provide a shared framework for interpretation across experimental and field settings. In parallel, immune readouts, protective thresholds, and key environmental modifiers are defined, monitored, and harmonized manner across studies. This stage is less focused on mechanistic depth and more on methodological alignment, ensuring that data generated across different systems is comparable and interpretable. It also supports more consistent regulatory assessment by improving the reliability and uniformity of measurement. Together, this tier represents the most immediately actionable steps based on current methodological capacity and existing surveillance frameworks.

5.2 | Medium-Term Priorities: Mechanistic Consolidation and Data Integration

As this framework becomes more established, the focus shifts toward mechanistic understanding. This stage involves the processes underlying vaccine-induced immunity, including immune memory formation and maintenance, antigen processing and presentation, activation of effector pathways, and the modulatory role of adjuvants. These processes are inherently context dependent and vary across species, pathogens, and environmental conditions. Accordingly, mechanism should be understood not as a single linear pathway but as

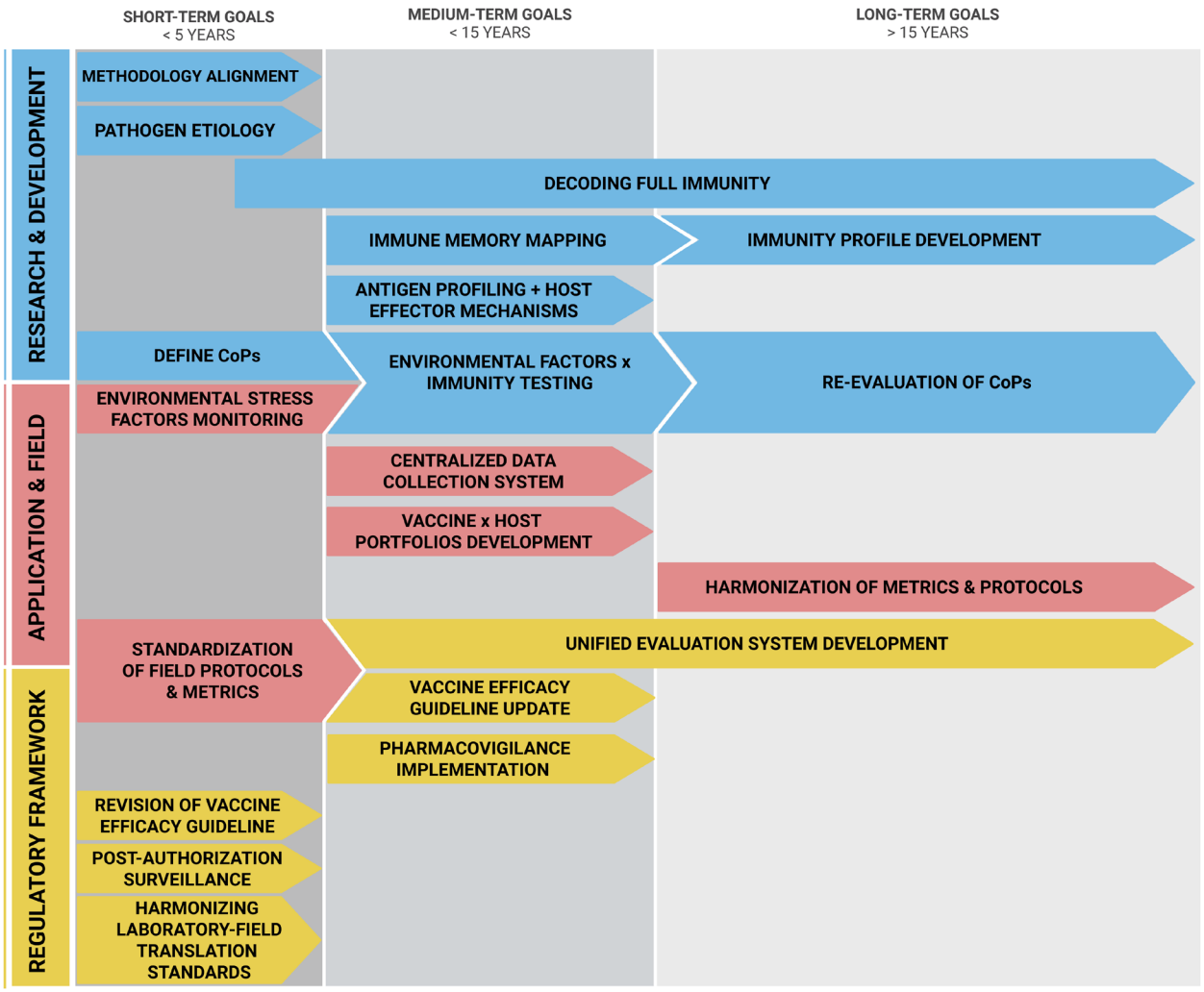

FIGURE 5 | A priority-driven roadmap from empirical ("Black-Box") vaccine evaluation toward predictive, mechanistically resolved frameworks. The sequential horizons represent progressive stages of implementation (short-, medium-, and long-term) and are based on approximate estimates of research translation rates as well as expected regulatory and methodological adoption timelines. CoPs: correlates of protection.

an interconnected network of biological interactions. In parallel, the need for integrated data structures becomes more pronounced, enabling the consolidation of infection dynamics, vaccination outcomes, and environmental variables into coherent analytical frameworks. Regulatory systems also evolve at this stage by incorporating environmental parameters, particularly temperature, as essential determinants of vaccine performance rather than secondary variables and thus outline these aspects in guideline protocols. However, this stage requires coordinated experimental and computational development but is feasible within current multi-omics and systems immunology approaches.

5.3 | Long-Term Priorities: Predictive and Regulatory Transformation

In the long term, the objective shifts from mechanistic description toward predictive capability. This phase represents a conceptual shift toward predictive vaccinology and depends on validated correlates of protection and integrated modeling frameworks. In this phase, validated CoPs are expected to function as reliable predictors of field efficacy. Immune signatures can be longitudinally tracked and linked to outcomes such as survival, immune recall dynamics, and durability of protection. Environmental variability is incorporated as an intrinsic component of predictive models rather than treated as confounding noise. This enables a transition in regulatory thinking from reliance on lethal challenge models toward non-invasive, performance-based evaluation frameworks. Computationally inspired decision-analytic approaches, such as decision-tree structures, may provide a scalable framework for organizing vaccine–pathogen–host interactions across diverse application contexts.

5.4 | Integrated Perspective Across Tiers

Across all stages, development should be viewed as a progressive refinement of system understanding rather than a discrete endpoint. Each step reduces uncertainty, improves cross-scale integration, and increases the predictive robustness of the

TABLE 1 | Overcoming hurdles in aquaculture vaccination: Key challenges, strategic solutions, and implementation pathways.

Challenge (current barriers)	Strategic solution	Likelihood of implementation	Optimal implementation strategy	Estimated cost
Mismatching efficacy laboratory vs. field	Comprehensive field trial validation	High—being implemented as we speak	Independent field auditing with standardized sentinel reporting.	Moderate to high: Requires multi-season monitoring and independent verification.
Lack of measurable correlates of vaccine efficacy in real world	Advanced high throughput immunology and vaccinology tools	Medium–high (technically feasible but biologically and logistically complex)	Prioritize mechanistic correlates over survival endpoints for transmission control.	High (requires multi-platform omics and bioinformatics infrastructure).
Environmental interference (Temp, O_2, water quality)	Optimized environmental control during vaccination	High—being implemented as we speak	Vaccination within species-specific thermal windows to optimize immune kinetics.	Moderate: Costs associated with water quality monitoring and thermal regulation infrastructure.
Handling and stress impact	Sedatives for IP, Oral/bath vaccines and/or boosting	High—being implemented as we speak	Bio-mitigation: Administer sedatives or psychobiotics during IP injection to suppress neuroendocrine and oxidative stress in gills.	Low to moderate: Cost of sedative agents or specialized mucosal delivery formulations.
Genetic diversity leads to host genotype—vaccine mismatch	Precision geno-matching of vaccines to specific populations	Low—prohibitively expensive and methodologically complex	Align vaccine strain selection with broodstock MHC class I/II polymorphism profiles.	Very high: requires large-scale genotyping and strain-matched vaccine production.
Protection development window mismatching the disease	Early-life stage vaccines; stimulation of trained innate immunity	Medium—methodologically complex	Ontogeny-targeted priming: delay vaccination until lymphoid maturation to prevent immune dysregulation.	Moderate: R&D for ontogeny-specific formulations and early-life delivery systems.
Systemic vs. mucosal misalignment	Oral/bath vaccines when mucosal protection is needed, oral/bath boosting	High—being implemented as we speak	Prime mucosal IgT+ memory via immersion or oral routes, followed by systemic IP boosting.	Moderate: investment in mucosal delivery platforms and antigen encapsulation technologies.
Fading immune memory and durability	Strategic oral/immersion booster regimes	High—being implemented as we speak	Strategic boosting: oral boosters during grow-out to sustain memory B cells and plasma cells.	Moderate: Ongoing costs for repeated administration and booster formulations.

framework. In this sense, the concept of a "White Box" should be interpreted not as a final achievable state but as an ideal goal that structures the continuous advancement of aquaculture vaccinology.

6 | Concluding Remarks

For decades, fish vaccinology has operated much like early cartography, successfully navigating by familiar landmarks of survival data while leaving the underlying mechanistic landscape largely uncharted. At present, vaccine performance emerges from a deeply entangled "Black Box" system where host biology, pathogen diversity, and environmental variability interact in ways that remain invisible to outcome-based metrics. Moving beyond this empirical layer requires a structured, multidimensional framework that redefines the vaccine itself while engineering tailored, practical solutions (Table 1). Extending these system-level limitations to a broader conceptual horizon raises a fundamental question about the future direction of vaccinology. While the next generation of vaccines will certainly evolve alongside technological advances, the true progress cannot be driven by tools alone. Without a structural understanding of the teleost biological architecture, even the most advanced platforms lack the leverage to disrupt the *status quo*. The path forward demands radical transparency in how vaccines interface with host networks. Future fish vaccines must move away from blind systemic stimulation and instead harness internal biological pathways, such as cellular communication networks and host-derived transport mechanisms, to autonomously guide antigens toward relevant immune niches, precisely orchestrating long-term memory formation. Ultimately, fish vaccines must evolve into accessible, predictable, and demystified tools. This trajectory mirrors human vaccinology, where demystification remains an ongoing pursuit. However, this transition also represents one of the most complex challenges ahead, requiring not only system-level understanding but also a rethink of how we think about and handle vaccines.

Author Contributions

Mikolaj Adamek: writing – original draft, writing – review and editing, funding acquisition. **Jiří Kyslík:** conceptualization, methodology, data curation, visualization, writing – review and editing, writing – original draft, investigation. **Tomáš Korytář:** conceptualization, funding acquisition, writing – review and editing, investigation, supervision.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available in the Supporting Information of this article.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Table S1:** List of field studies performer on vaccines against bacterial and viral pathogens. IM: intramuscular delivery; IP: intraperitoneal delivery; RPS: relative percentage survival.