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Understanding the assessment of vitellogenin and yolk protein immune-relevant and antioxidant activities in teleost

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Abstract
Owing to their functions in the prevention of chronic diseases and the use of preservatives in food and cosmetics, antioxidant agents have gained much interest in recent years. Vtg and its related Pv also bear antioxidant anti-ROS activities. These proteins, being components of our food source, are therefore natural antioxidants. They can be an important antioxidant with potential for food and cosmetics preservation as well as for the stabilisation of chronic disease states. Vitellogenin (Vtg), the main precursor protein of egg yolk, is historically thought to provide protein- and lipid-rich nutrients to grow. However, Vtg 's roles extend beyond nutritional functions, as well as its related yolk proteins lipovitellin (Lv) and phosvitin (Pv). Accumulating evidence has shown that Vtg, Lv, and Pv engage with multifaceted roles in host innate immune response. They all can serve as multivalent receptors for pattern recognition capable of recognising invading microbes. Even Vtg and Pv can serve as immune Protection with multifaceted capabilities. They all can serve as multivalent receptors for pattern recognition capable of recognising invading microbes. Also, Vtg and Pv can serve as immune effectors which can destroy bacteria and viruses. In addition, Vtg and Lv as opsonins are shown to possess phagocytosis-promoting activity. Besides these immune-relevant functions, Vtg and Pv have antioxidant activity which can protect the host against oxidant stress. These non-nutritional functions clearly expand our understanding of the molecules' physiological roles, thus providing a solid foundation for the future application of the molecules in human health.

Keywords: Antioxidant activity, vitellogenin, lipovitellin, phosvitin, immunity

1. Introduction
Most fish are oviparous, with outward fertilising of their eggs [1]. The final result of oocyte growth and differentiation is the eggs or haploid reproductive cells, which develop into viable embryos after fertilization [2]. In general, the development of oocytes includes several steps: the creation of primordial germ cells (PGCs) and the transformation of PGCs into oogonia, and then into oocytes. Thereafter, during vitellogenesis, significant maternal information and molecules required for early embryo development are deposited in growing oocytes, including RNAs, proteins, lipids, vitamins, and hormones [2, 3]. One of the most significant proteins stored in oocytes is vitellogenin (Vtg), a superfamily member of the broad lipid transfer protein (LLTP) [3, 4, 5]. Vtg is a high molecular mass glycolipophosphoprotein, which typically circulates as a homodimer in the blood (vertebrates)/hemolymph (invertebrates) [4, 6, 7, 8]. In a given species, there are normally many Vtg isoforms, which are encoded by a multigene family [9, 10]. In chicken *Gallus gallus* [11, 12], four in Africa frog *Xenopus laevis* [13, 14] and six in nematode *Caenorhabditis elegans* [15], for example, three Vtg genes have been identified. In teleosts even multiple vtg genes are common. In zebrafish *Danio rerio* [16, 17], two vtg genes in carp *Cyprinus carpio* [18], four vtg genes in medaka *Oryzias latipes* [10], three vtg genes in striped bass *Morone saxatilis* [19], and three vtg genes in white perch *Morone americana* [20] are identified. Almost all vellogenins (Vtgs) processed by genetic factors get a similar approach in vertebrate species, like fish and invertebrates, particularly insects [21, 22]. In certain circumstances, Vtg comprised of 3 preserved regions, LPD N (as well recognized as vitellogenin N or LLT domain), located just at N-terminus, the unknown function domain (DUF) 1943, as well as the von Willebrand factor type D domain (vWD), situated only at C-terminus and spread through a broad range of proteins [21]. Or sometimes, in some Vtg proteins in vertebrates like fish and chicken, the undefined role domain called DUF1944 is believed to undergo among DUF1943 and vWD.

Beginning just at N-terminus, the complete Vtg fish comprises of a polypeptide chain, a lipovitellin heavy chain (LvH), a phosphorylated serine rich phosvitin (Pv), a lipovitellin light chain (LvL) as well as a β-component (β-C) plus a vWD-coded C-terminal region (CT) [4, 19, 20, 23].

In particular, some teleostean Vtgs lack Pv and most of the carboxyl-terminus (β-component and C-terminal peptide), consisting only of LvH and LvL [23]. Also, Pv is absent in most Vtg invertebrates [8, 16]. In the females of nearly all oviparous animals, including fish, amphibians, reptiles, birds, most invertebrates and platypus, Vtgs, the precursors of egg yolk proteins, exist. Vtgs are normally synthesised in extra-ovarian tissue (in vertebrate liver, crustacean hepatopancreas and fat body of insects) and transported through the circulation system to the ovary where they are internalised into rising oocytes through receptor-mediated endocytosis during vitellogenesis with diverse proportional composition [2, 7, 19, 24, 25, 26, 28, 29, 30, 31]. Interestingly, the concentrations of various Vtgs internalised by increasing oocytes are not always equal to the concentrations of circulating Vtgs in the blood, which may be due to the control of the system of multiple ovarian receptors engaged in different Vtg endocytosis [32, 33, 34, 35]. When internalised in the oocytes, the aspartic protease cathepsin D cleaves Vtgs proteolytically to create yolk proteins, such as Lv subunits, Pv and β-C [36, 37, 38, 39, 40, 41, 42, 43]. Lv subunits and Pv are contained in yolk globules or platelets while β-C remains a soluble fraction in cytoplasm [44, 45, 46]. Lv, the largest yolk protein derived from Vtgs proteolytic processing, is an apoprotein that mainly delivers phospholipids into oocyte growth [36, 47]. Pv, the smallest yolk protein, consists primarily of phosphorylated serine residues expected to stabilise nascent Vtg structure during lipid loading and improve blood solubility of Vtg [4, 47]. β-C and CT, small vWD cleavage products with a highly conserved pattern of repeated cysteine residues, are intended to stabilise Vtg dimer for cell recognition and receptor binding and to protect Vtg or its component yolk proteins against premature or inappropriate proteolysis [4, 19, 20]. All these yolk proteins are later used as nutrients in the development of embryos to feed their cells [48, 49].

Vtgs were once regarded as a female-specific protein [50, 51]; however, synthesis has been shown, although in smaller amounts, to occur in male and even sexually immature animals [52, 53, 54], indicating that Vtgs presumably serve a more general function independent of the sex. Recently, it has been shown that both Vtgs and yolk proteins are related to the immune response and antioxidant activity in fish, questioning the conventional view that Vtgs and yolk proteins are a basic source of nutrients for developing embryos. The immune-relevant and antioxidant activities of Vtgs and yolk proteins in fish are listed below.

2. Immune Roles of Vtgs

Gathering data revealed many non-nutritional functions for Vtg. In the honeybee *Apis mellifera*, an advanced eusocial insect [55, 56, 57, 58], for example, Vtgs were shown to be correlated with social organisation, temporal division of labour and foraging specialisation, control of hormonal dynamics and change in gustatory responsiveness. Recent studies show that Vtgs also perform roles important to the immune system. Zhang *et al*. observed that Vtg purified from the ovaries of the protochordate amphioxus (*Branchiostoma japonicum*) exhibited hemagglutinating activity against chick, toad and grass carp erythrocytes as well as antibacterial activity against the Gram-negative bacterium *E Coli.* The first solid evidence showing that Vtg preforms an immune-relevant function [59]. Eventually afterwards, this was observed which Vtg distilled from of the *Puntius conchonius* rosy barb could inhibit the growth of the Gram-negative bacteria *E. Coli*, *E. Aerogenes* and *Pseudomonas putida* and Gram-positive bacteria *Staphylococcus aureus*, Bacillus *subtilis* and *Streptococcus pyogenes* [60] and the carp Vtg that can inhibit its development of *E. Coli* and *S. aureus* in a dose-dependent manner [61]. Interestingly, protostomal Vtgs also tend to exhibit antibacterial activity. It has recently been shown that Vtg from the scallop (*Patinopecten yessoensis*) has an antibacterial activity against Gram-positive and Gram-negative bacteria [62]. In addition, Vtg is in nematode *C. elegans* also tends to be active in its antimicrobial safety. Decreased survival was found in Vtg-knockdown *C. elegans* following infection with the pathogen [63]. A further justification for all of this an invertebrate Vtg function linked to tolerance to bacteria have been produced by enhancing nematode resistance to the pathogen *Photorhabdus luminescens*, when oestrogen 17 β-estradiol and phytoestrogen daidzein stimulated the development of Vtg. Reduction of Vtg caused by soy isoflavone genistein, however, decreased the host resistance to *P. luminescens* [64]. Brought along, the antibacterial activity it seems to be a universal property of both vertebrate and invertebrate Vtgs.

Vtg is historically assumed to even provide protein and lipid-rich components for both the growth of embryonic and worms. Even so, that accumulation of data shows that this position goes even beyond nutritional purpose. In developed eusocial pollinator species, have shown that the Vtgs is correlated to social organisation, temporary division of labour and specialisation in foraging, control of hormonal processes and alteration of taste sensitivity. Latest experiments have shown that Vtgs also perform immune-relevant functions. Vtg is able to identify infectious pathogens as just a multivalent information processing receptor that destroys bacterial or neutralises the virus as an effector molecule and stimulates phagocytosis as opsonine. In addition, Vtg also shows behaviours for hemagglutinate erythrocytes and aggregate pathogens. In relation with immune functions, Vtg performs a new function as just an antioxidants. Shi *et al*., showed that intraperitoneal injection of *E. coli* was able to enhance the level of serum Vtg in male *P. conchonius* [60]. This has recently been confirmed by Lu *et al.*, who showed that expression of vtg genes in the skin of zebrafish was induced following the challenge with Gram-negative bacterium *Citrobacter freundii* [65, 66].

Such results suggest that Vtg first acts as just a multivalent pattern recognition receptor able to recognise aggressive Gram-negative and Gram-positive bacteria and also fungi and is active in host immune response also as scanner. In a recent analysis pursuing PGN identification proteins in giant tiger shrimp (*Penaeus monodone*), 83 kDa proteins were extracted from an *in vitro* PGN pull-down binding assay and classified as Vtg-like protein by mass spectrometry along with Western blots with Vtg-specific monoclonal antibodies recorded from *P. monodone*. [70], involving the Vtg invertebrate also may play an important role in the information processing of receptors. Scanning electron microscopy and also bacterial cell experiments and protoplast analysis revealed that *H. otakii* Vtg was able to kill harmful bacteria by liquefying entire cells (with cell walls) rather than just protoplasts (without cell walls) by LPS and LTA interactions [71]. They

propose the Vtg functions as that of an effector molecule able to kill bacteria immediately. It was also noteworthy to note that Vtg was capable to facilitate the phagocytosis of microbes by macrophages. Li *et al*. mentioned *H. otakii* for the first time; Vtg might promote the engulfing of the *E. coli*, *S. aureus* and *P. pastoris* microbes by head-kidney-derived *in vitro* macrophages [69]. After, carp Vtg was found to have related phagocytosis-promoting activity [61]. With the on-site analysis of the effects of urban untreated sewage on freshwater mould *Elliptio complanata*, it was found the manufacture of Vtg-like protein was closely correlated with phagocytosis [72], proposing the relationship among Vtg and phagocytosis in invertebrates. In addition, *H. Otakii* Vtg was shown to be able to bind to a cell surface of macrophages and also not to those of RBC (red blood cells) [61, 69]. Collectively, these findings suggest that Vtg is indeed an opsonine which acts serves as a bridge molecule among the host macrophages and invasive microbes, resulting in increased phagocytosis. In particular, Liu *et al*. have identified which the *H. Otakii* Vtg is able to typically focused *P. pastoris* fungus for phagocytosis by macrophages extracted by *Lateolabrax japonicas* sea bass, suggesting this Vtg wasn't really species-specific [73]. Even more study found that Vtg-opsonized phagocytosis had typical characteristics of type I phagocytosis, like extension of the pseudopod, reliance on tyrosine kinase, and up-regulation of tnf-α and il-1β pro-inflammatory cytokine genes [73]. As a consequence, Vtg is indeed an information processing receptor susceptible of recognising microbes, a bacterial molecule capable of disrupting bacterial cell walls, and an opsonine capable of facilitating phagocytosis of pathogens by macrophages. Vtg's interdisciplinary immune-relevant behaviours are partly equipped with its multiple realms. Sun *et al.* stated that DUF1943 and DUF1944, and even some vWD, contributed to the role of Vtg as an information processing receptor, and that DUF1943 and DUF1944 (but not vWD) also contributed to the role of Vtg as opsonin [21].

Recent times, Garcia *et al*. have demonstrated the Atlantic salmon Vtg has a neutralising capacity against contagious pancreatic necrosis virus [74], indicating that Vtg also is active in target anti - viral resistance. This is further confirmed by the finding that mosquito (*Anopheles gambiae*) Vtg became able to interact with the anti-plasmodium response [75]. Such suggest the Vtg does have anti - viral function alongside antibacterial activity, this will require a thorough analysis throughout the coming years.

3. Immune functions in yolk proteins

Lv as well as Pv seem to be the primary yolk proteins produced by Vtg's proteolytic processing. Since Vtg is also an immunocompetent enzyme, hypothesising that Lv and Pv both have equal immune response is therefore rational. This hypothesis was first tested by Zhang and Zhang [76]. They demonstrated that the native Lv purified from ovulated eggs of the rosy barb *P. conchonius* was able to interact with LPS, LTA and PGN, as well as *E. coli* and *S. aureus*, but not with self-molecules such as the egg extracts prepared, indicating that Lv is a molecule capable of recognizing non-self components. In addition, Lv's bacterial binding activity allowed strengthening it macrophage phagocytosis of the bacteria, indicating that Lv is also a functional opsonin in the development of embryos / larvae [76]. Similarly, Pv was also shown to play a critical role in the immunity of zebrafish embryos via acting as a pattern recognition receptor and an antimicrobial effector molecule [77]. In line with this, hen egg yolk Pv was also shown to be able to inhibit the growth of the Gram-negative bacterium *E. icoli* and the Gram-positive bacterium *S. aureus* under thermal stress [78, 79]. Noteworthy, Pv 's affinity to LPS allowed the protein to neutralise endotoxin, promoting the endotoxemia mice survival rate [79]. It was recently shown that a truncated Pv (Pt5) consisting of the *C*-terminal 55 residues of zebrafish Pv also displayed similar immune activities with Pv, including antimicrobial activity against *E. coli*, *Aeromonas hydrophila* and *S. aureus*, and specific affinity to LPS, LTA, and PGN [77].

Through intraperitoneal injection of such a Pv-derived peptide, the rate of survival of the zebrafish is pathogenic A could increase. Hydrophila as well as the amount of pathogens in different tissues to decrease markedly, indicating that Pt5 may prevent the multiplication / dissemination of the pathogen as just an antimicrobial in the host. In addition to direct antimicrobial activity, Pt5 was also shown to be able to regulate the host immune responses via suppressing the expression of pro-inflammatory cytokine genes (*il-1β*, *il-6*, *tnf-α* and *ifn-γ*) and simultaneously enhancing the expression of anti-inflammatory cytokine genes (*il-10* and *il-4*), suggesting a dual role of Pt5 as both immune effector and modulator [80]. Recently, a mutant peptide of Pt5 (designated as Pt5e), generated by site-directed mutagenesis, was shown to have stronger bactericidal activity and LPS-neutralizing activity [81].

In addition, Sun *et al*. have also shown that recombinant zebrafish Pv seems to be able to inhibit cytopathic effect formation in cells infected with lymphocystis disease virus (LCDV) and decreasing the quantity of viruses in highly contagious cells along with contaminated zebrafish, suggesting that Pv possesses an antiviral activity and participates in immune defense of host against the infection by viruses like LCDV [82]. Taken together, these data show that like Vtg, Lv and Pv are both immune-competent molecules involved in immune response of the host against invading pathogenic microbes.

In contrast with immune functions, the antioxidant function is another novel role of Vtg. This was first seen with Ando and Yanagida that Vtg from *Anguilla japonica* (eel) was willing to tolerate the copper-induced oxidation and might shield the very low energy density of lipoprotein (VLDL) from copper-induced oxidation [83]. It was the first assessment to note which Vtg must have antioxidant capacity and is used to inhibit free-radical reactions in oocytes of fish. Related antioxidant capacity has also been proposed for nematode (*C. elegans*) Vtg [84]. In honeybee, Vtg has been shown to be able to suppress oxidative stress by scavenging of free radicals, thereby rising the lifetime in optionally sterile castes of workers and reproductive queen castes [85, 86]. The honeybee Vtg was also demonstrated in a recent study to be capable of recognizing cell damage through its binding to membrane and shielding living cells from damage by reactive oxygen species (ROS) [87]. It's clear that in both invertebrates and vertebrates, Vtg protects the cells from damage to ROS.

Hen-egg yolk is well established Pv, as Vtg-derived major protein, show good antioxidant activity due to its high content of serine and phosphorus, that makes this protein one of the most potent chelating iron agents [88, 89, 90]. Very recently, we showed that zebrafish recombinant phosvitin (rPv) was an antioxidant agent capable of inhibiting the oxidation of the linoleic acid, and scavenging the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical. We also showed that zebrafish rPv is a cellular antioxidant capable of protecting radical-

mediated oxidation of cellular biomolecules. Importantly, zebrafish rPv is non-cytotoxic to murine macrophages RAW264.7 [91]. These findings indicate that Pv is also a potent antioxidant in fish. If Lv, another essential protein derived from Vtg, any antioxidant activity remains available, something worth investigating.

4. Potential uses in the area of public health

Antibiotics are widely used globally to manage microbial infections in clinical practise, but cases of resistance have been reported to the majority of antibiotic groups, which has become a serious challenge to human health in many parts of the world [92, 93]. It is thus essential to develop new antibiotic agents to combat these resistant pathogens. Antimicrobial proteins/peptides (AMPs) are potential candidates to solve this problem. As a protein/peptide with antimicrobial activity widely present in plants, animals and microbes, AMP commonly is a cationic and amphipathic molecule with a net positive charge and a high percentage of hydrophobic residues [94]. These structural features provide AMP with the ability to interact with microorganisms' anionic cell wall and phospholipid membranes, which makes resistance production more difficult for pathogens [95]. Vtg and its derived protein Pv from oviparous species, especially teleost fishes, both display antibacterial activities with a broad antibacterial spectrum [59, 60, 61, 62, 68, 71, 77, 78, 79], and hence can be used as pro-drug to develop novel antibiotic agents. For example, a single or double mutagenesis produced a total of six mutant peptides based on the residual sequence of Pt5, the C-terminal peptide of zebrafish Pv; among these, a mutant named Pt5e showed stronger antibacterial activity against *E. coli* and *S. aureus* [81], and was able to kill five strains of multiple drug resistance bacteria isolated from clinical cases via disturbing their cell membrane integrity [96, 97, 98, 99].

5. Conclusions

Vtg, the forerunner of major egg yolk proteins, is historically thought to provide protein- and lipid-rich nutrients for embryo and larvae development. However, the collection of evidence suggests that Vtg and its related proteins Lv and Pv also have non-nutritional functions: they are not only active in immune response but also in antioxidant reactions. These non-nutritional functions explicitly enhance and expand our knowledge of the biochemical roles of molecules and, at the same time, offer a solid foundation for the future application of the molecules in human health.

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