Fakulta rybářství
a ochrany vod
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of Waters

Jihočeská univerzita
v Českých Budějovicích
University of South Bohemia
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Doctoral thesis

Susceptibility of common carp strains to the disease caused by carp edema virus

Ali Asghar Baloch

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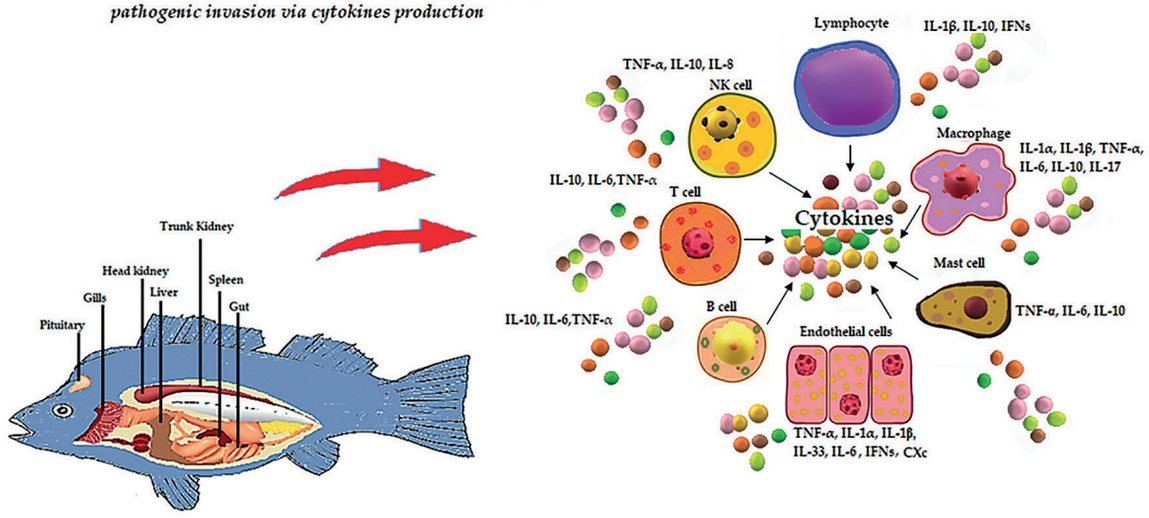

Doctoral thesis by
Ali Asghar Baloch

Czech Republic, Vodňany, 2023

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CONTENT

CHAPTER 1

GENERAL INTRODUCTION

1. Introduction

Fishing industry, including aquaculture (farming of aquatic organisms), is facing a widespread threat from diseases that can infect fish. These diseases have the potential to cause significant damage not only at the local level, but also regionally and internationally. In other words, the impact of these diseases on the fishing industry could be significant and far-reaching. As the world's population grows and demand for fish as a food source increases, traditional marine fishing methods may not be able to keep up. This is where aquaculture, or fish farming, comes in as a potential solution. In aquaculture, the susceptibility of farmed fish to diseases is a major constraint, whether caused by husbandry practices or external factors such as pollution, microorganisms, climate change, or even changes in the dynamics of supply and demand (Yancey et al., 2014). Diseases caused by pathogens are of uttermost importance because they may induce heavy mortality. The presence of viruses in fish pathology has been known since the pre-1950s (Wolf, 1988) though the actual causative agents of the diseases was not proven until the technique involving the isolation of piscine viruses in fish cell lines was established (Wolf and Quimby, 1962; Wolf and Malsberger, 1966). This technique of isolation of fish viruses *in vitro* became the yardstick for the detection of viruses in various species of fish such as the Atlantic salmon, rainbow trout, carp, catfish and among others. In natural conditions, the diseases caused by some pathogens are mild as observed in the case of herpes virus infections (Hanson et al., 2011). The nature of such diseases, however, is worse in immune-compromised hosts. Fish may become immune-compromised due to high-density rearing conditions in tanks or ponds, which can cause stress and lead to competition for resources and the spread of diseases. Poor water quality, including high ammonia levels and low oxygen concentrations, can also contribute to immune compromise and increase susceptibility to infections. Controlling viral infections in fish is a challenging task due to the unavailability of effective treatment options. As such, preventive measures are often the primary means of controlling and mitigating the risk of disease outbreaks in aquaculture settings. Therefore, in order to reduce aquaculture industry losses, it is essential to better understand and characterize all the intervening factors that lead to a disease outbreak. Since the beginning of the 20th century, significant research efforts have been made to understand fish diseases, their causes and pathogenesis. The findings of these studies have provided new tools for detecting, treating, and controlling fish diseases.

A major contributor to global freshwater fish production is the common carp (*Cyprinus carpio L.*) (FAO, 2013), however, it may also contribute to the spread of a wide range of fish diseases since many of them are dispersed through trade in common carp both locally and internationally. There is also a very lively international trade in ornamental fish, where the ornamental variety of carp – Koi – dominates. The interest in rearing Koi (*Cyprinus rubrofuscus*), has been stimulated by its high economic value and increasing market demand, rendering it one of the most popular ornamental fish (Domasevich et al., 2022). Many years of selection to achieve desirable color patterns have resulted in reduced natural resistance in koi and therefore their increased susceptibility to disease. Furthermore, the lack of knowledge among hobbyist aquarists about fish diseases and preventive measures, coupled with a lack of formal training in fish management and health, may contribute to the dissemination of infections. Many viral, bacterial, and parasitic diseases are prevalent in common carp. Due to high mortality, viral infections were found to be the main reasons for drastic economic losses in the production of common carp (Jeney and Jeney, 1995). In aquaculture systems, due to the high concentration of fish, they are easy to disseminate from fish to fish. Fish surviving viral infection can act as persistent carriers, continuously spreading the viruses throughout the aquatic environment, and contaminating other fish. Therefore, effective measures are needed to control viral diseases. Characteristics of significant viral diseases of cyprinid fish are summarized in Table 1.

Table 1. *Basic attributes of important viral diseases in common carp (Cyprinus carpio L.) (Adopted and changed from https://www.oie.int/ and https://edis.ifas.ufl.edu/).*

	Carp Edema Virus Disease	**Koi Herpesvirus Disease**	**Spring Viremia of Carp**	**Carp Pox**
Synonyms	Koi sleepy disease	Carp interstitial nephritis and gill necrosis virus disease	Infectious dropsy of carp	Carp pox; Herpesviral epidermal proliferation in carp; Herpesvirus septicemia in carp (HSC)
Abbreviation	KSD/CEVD	KHVD, CNGVD	SVC	HEPC; HSC
Viral agent	Carp edema virus (CEV), family Poxviridae, DNA virus	Cyprinid herpesvirus 3 (CyHV-3), family Alloherpesviridae, DNA virus	Carp sprivivirus, family Rhabdoviridae, RNA virus	Cyprinid herpesvirus 1 (CyHV-1), family Alloherpesviridae, DNA virus
Species susceptible	common carp; koi	common carp; koi; other species may carry virus	common carp; koi; other species from Cyprinidae, Esocidae, and Acipenseridae	common carp; koi
Optimal water temperature	59–77 °F (15–25 °C) & (6–10 °C)	64–81 °F (18–27 °C)	41–64°F (5–18 °C)	<68°F (<20 °C)
transmission	Direct contact; infected water/mud, equipment; vectors	Direct contact; fecal material; infected water/mud, equipment; vectors	Direct contact; fecal material; infected water/mud, equipment; vectors	Direct contact; fecal material; infected water/mud, equipment; vectors
Age susceptibility	All age groups of common and koi carp.	All age groups of fish; under experimental infection, younger fish (up to 1 year of age) are more susceptible to infection.	All age groups of fish; young fish up to 1 year of age are more susceptible.	Young fish (under 2 months) are more susceptible than mature.
Clinical signs -behavioral	Unresponsiveness and lethargy, lying motionless on their sides at the bottom of the tank, unless get touched	Lethargy; swimming close to the surface; respiratory distress; erratic behavior	Lethargy; swimming low in tank or pond bottom; awkward swimming	None
External	Extensive erosions or hemorrhages of the skin with edema (swelling) of the underlying tissues, sunken eyes (enophthalmos)	Gill necrosis; sunken eyes; notched nose; secondary bacterial and parasitic infections	Exophthalmia; pinpoint skin hemorrhage; abdominal distention; swollen vent, inflamed and trail mucoid casts	Smooth raised wart-like skin lesions

Internal	Pale gills and gill arches with multifocal areas of white discoloration	Adhesions in the abdominal cavity, enlarged kidney and liver with petechial haemorrhages	Edema; inflammation; pinpoint hemorrhages of many organs including swim bladder	None
Testing Methods	Direct method (PCR assays)	Direct methods (virus isolation and PCR); indirect methods (ELISA)	Direct methods (virus isolation and PCR)	Direct methods (virus isolation and PCR assay)
Fish serve as carrier	Yes	Yes	Yes	Yes
Regulatory Status	Not notifiable for EU or OIE	Notifiable for OIE with no mandatory consequences	Notifiable for OIE with mandatory consequences; import regulations	Not notifiable for EU or OIE
Treatment	None	None	None	None
Prevention/ Control	Depopulate infected stocks; practice good biosecurity; purchase fish from a known reputable source, new fish should be quarantined for a minimum of 30 days	Depopulate infected stocks; practice good biosecurity; purchase fish from known reputable source; keep susceptible species separated; new fish should be quarantined for a minimum of 30 days	Depopulate infected stocks; practice good biosecurity; purchase fish from known reputable source; keep susceptible species separated; new fish should be quarantined for a minimum of 30 days	Depopulate infected stocks; practice good biosecurity; purchase fish from known reputable source; new fish should be quarantined for a minimum of 30 days
Disinfection of equipment	Salt baths (0.5%) as supportive therapy for infected fish to manage CEV symptoms. Whilst salt baths reduce stress levels and slow down the spread of the virus, it does not cure it. (Seno et al., 2003, Miyazaki et al. 2005, Way and Stone, 2013)	Chlorine (200 ppm for 1 hour) and quaternary ammonium compounds (500 ppm for 1 hour).	Chlorine (500 ppm for 10 minutes); ozone; gamma/UV radiation; pH 10.0; heat 60°C for 15 min.	Chlorine (200 ppm for 1 hour); quaternary ammonium compounds (500 ppm for 1 hour).

1.1. Carp edema virus disease (CEVD) or koi sleepy disease (KSD)

1.1.1. CEV taxonomy and its genetic lineages

Taxonomically, CEV belongs to the family Poxviridae which includes viruses with double-stranded linear DNA (Jung-Schroers et al., 2015). It is described as an enveloped virus and appears as a mulberry-like structure ranges 250–280 nm in diameter (Figure 1) (Oyamatsu et al., 1997). Through transmission electron microscopy, poxvirus like particles were observed in the cytoplasm of hypertrophic epithelial cells of the secondary gill lamellae (Ono et al., 1986; Miyazaki et al., 2005; Hesami et al., 2015). The cytoplasm of infected gill epithelial cells was later found to contain ovoid virions measuring 360 nm in diameter and flattened on one side (Hedrick et al., 1997). Overall, poxviruses are one of the largest viruses in terms of their size range of 250–400 nm.

The CEV genome contains 460 kbp and encodes about one hundred proteins arranged into four structural groups: the core, the membrane, the lateral body, and the envelope (Gjessing et al., 2015; Mekata et al., 2021). Genetic analyses of CEV only rely on the p4a gene analysis, encodes the putative major core protein, due to a lack of data on the complete virus genome sequence (Adamek et al., 2017a; Matras et al., 2017). Due to the 6% to 10% variation in p4a gene sequences, CEV is classified into three genogroups called I, IIa, and IIb (Adamek et al., 2017a). Common carp are the primary host of genogroup I, which has been detected in most European waters. A majority of genogroup IIa reports have been in koi, while genogroup IIb has been detected in both carp and koi samples (Matras et al., 2017; Adamek et al., 2018a; Matějíčková et al., 2020; Ouyang et al., 2020). Recent reports have identified two new genotypes of CEV, IIIa and IIIb. In the study, the Austrian isolates were compared with three already known CEV genotypes (I; IIa & IIb) to understand their phylogenetic relationships (Soliman et al., 2019). There was, however, a disagreement over the addition of two new Austrian CEV genotypes, since the two additional genotypes were the result of an analytical error. As a result, the sequences for these proposed new Austrian CEV genotypes, which were submitted to Gene Bank, were in reverse order and belonged to the already established and existing genotypes I and II (Zrnčić et al., 2020).

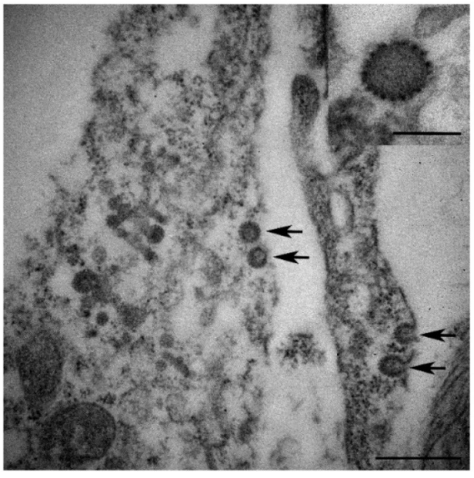

Figure 1*. Electron microscopy of gills from CEV-affected koi. Few number of virions present in the cytoplasm (arrows). Scale bar = 500 nm. (Inset) Virion showing electron dense core with surface membrane worn by surface globular units. Scale bar = 200 nm. (Obtained from Pikulkaew et al., 2020)*

1.1.2. Global distribution

In the 1970s, Japanese scientists documented a case of carp edema, a viral edema that affected juvenile koi carp. Based on the observations, they named the causative agent as carp edema virus, presuming it to be the cause of the disease, which presented as an edematous syndrome (murakami et al., 1976; Ono et al., 1986). In the past, CEVD outbreaks were only observed in koi populations with mortality rates up to 99%, hence it is called koi sleepy disease (KSD), but later it has been diagnosed also in common carp of all ages with high mortality rates (Haenen et al., 2016). After being discovered, till to date, infection caused by carp edema virus is gaining attention, as it is characterized by high mortality rates but can be also manifested as subclinical infection (Amita et al., 2002; Ono et al., 1986). After its initial detection in Japanese koi populations, CEV has spread worldwide with cases detailed in nations like the United States (Viadanna et al., 2015), Hungary (Hedrick et al. 1997), Czech Republic (Vesely et al., 2015; Matějíčková et al., 2020), France (Lovy et al., 2018), Netherlands (Haenen et al., 2014), Italy (Pretto et al., 2013), Germany (Jung-Schroers et al., 2015; Bachmann and Keilholz, 2016), England (Way and Stone, 2013), India (Swaminathan et al., 2016), Poland (Matras et al., 2017), Austria (Lewisch et al., 2015), China (Ouyang et al., 2018), and South Korea (Kim et al., 2020). In addition, it spreads to Iran (Ziafati et al., 2022), Iraq (Toffan et al., 2020) and Thailand (Pikulkaew et al., 2020).

1.1.3. Clinical signs and gross pathology

Lethargic behaviour is one of fish's most prominent external symptoms of active CEV infection. This is evident in their inactivity and sluggishness, hence the name "koi sleepy disease". Therefore, they can be seen lying in the bottom of tanks for extended periods. Fish with disease swim for a moment, then take a position on the side again when touched (Miyazaki et al., 2005). There may be pale oedema of the gills due to epithelial hyperplasia of the filaments; enophthalmia; hemorrhages around the mouth or the base of the fins; and anal edema, which can also be associated with KSD (Figure 2, 3) (Oyamatsu et al., 1997; Miyazaki et al., 2005; Pretto et al., 2013; Zhang et al., 2017). A large amount of mucus is produced on the skin and gills as well (Zhang et al., 2017). There is an increased size of some internal organs in KSD-affected fish, including the spleen, kidneys, and heart. It is observed that the gastrointestinal tract appears edematous and contains no intestinal content (Lewisch et al., 2015). Subsequently, it is possible that these gross lesions may lead to death of the infected fish. The fish starts dying usually between 6 and 7 days post-infection in water temperature between 20 and 24 °C (Zhang et al., 2017). It has also been reported that disease outbreaks also occur in common carp and sometimes even in koi during periods of low water temperature (6–10 °C) and that the disease is more protracted with a lower mortality rate in these cases (Way and Stone, 2013; Lewisch et al., 2015). The distinction between CEV and Cyprinid herpesvirus 3 (CyHV-3) infections is arduous to establish solely based on the pathologies elicited by these agents (Jung-Schroers et al., 2015; Ouyang et al., 2020). Because of this, diagnosing a diseased fish based on its clinical signs should be done with great care.

Figure 2. *Gross lesions in infected koi. Koi displaying extensive petechial bleeding points on skin especially in the abdomen region. Hyperemia at the base of the caudal fin can also be seen.*

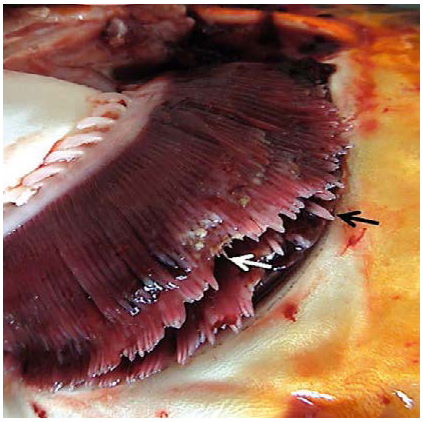

Figure 3. *Gills of carp edema virus-infected koi. Swelling of the primary filaments (black arrow) and necrosis of gill tissue (white arrow) can be seen. (Obtained from Jung-Schroers et al. (2015), original publisher: BioMed Central)*

1.1.4. Scientific approach for CEVD/KSD diagnosis

Clinical signs and history of infection can be used to make a preliminary diagnosis of KSD. Gross clinical signs such as lethargy, slow swimming, suffocation of the fish and congregation at the water surface can occur during CEVD. However, these signs can also indicate oxygen deficiency, intoxication or a damage of gill tissue by parasites, so they do not necessarily signal this disease. It is also difficult to determine the difference between CEVD and other infection diseases, such as Cyprinid herpesvirus 3 (CyHV-3) or *Flavobacterium branchiophilum* infections (Jung-Schroers et al., 2015; Adamek et al., 2018b; Ouyang et al., 2018).

So far, CEV cannot currently be detected by re-isolating in cells because it is not feasible to cultivate CEV *in vitro* with currently available fish cell lines (Adamek et al., 2017b, 2018a; Jung-Schroers et al., 2015). Fathead minnow (FHM), Cyprinus carpio koi fin (CCKF), pearl spot fin (PSF), catopra fish fin (CFF), common carp brain (CCB), Horabagrus brachysoma fin (HBF), goldfish fin (GFF), angelfish fin (AFF), and numerous other teleostean cell lines have all been tried for CEV isolation (Jung-Schroers et al., 2015; Swaminathan et al., 2016). But neither the cytopathic effect (CPE) was observed, nor the virus could be isolated from any of the

aforementioned cell lines. The unavailability of a standard procedure for the isolation and amplification of CEV in cell lines has somewhat hampered further research in the field of pathogenesis, diagnosis, prevention, etc.

Further, histological examination was also used for the detection of CEV in infective tissue. An examination of the histology of the gill lamellae typically reveals edema in the respiratory epithelium and hyperplasia in the interlamellar epithelium. However, these changes alone are insufficient to make a definitive diagnosis (Ono et al., 1986). It may be possible to obtain additional evidence using the histology of infected organs. Use of histopathological technique also supports CEV diagnosis by using stained slides prepared from formalin fixed skin and gill tissues for observing microscopic abnormalities (Oyamatsu et al., 1997; Miyazaki et al., 2005). CEV-infected fish exhibit various symptoms, such as erosive and ulcerative lesions on the skin, accompanied by loose connective tissue and dilated vessels exhibiting edema (Miyazaki et al., 2005). It was evident from the histopathological changes of gill tissue sections that the fish had lost the typical anatomical organization, synechiae were present between adjacent gill lamellae, eosinophilic granular cells were infiltrated, and inflammatory exudate was present (Zhang et al., 2017). For detecting virus particles, transmission electron microscopy can be used to confirm the presence of CEV in gill tissue, however the method's degree of efficacy differs significantly between laboratories. According to research using electron microscopy, immature virions can measure up to 450 nm in diameter, whereas mature ones are generally oval-shaped and measure 400–413 nm in size (Hedrick et al., 1997; Oyamatsu et al., 1997).

Multiple researchers have developed various PCR-based detection methods (assays) for the detection of CEV-specific DNA at this time. The sequences of CEV DNA fragments encoding protein p4a were used to create each of those distinct PCR-based detection techniques (Adamek et al., 2017b). Routine screening of clinically healthy fish is recommended to detect latent carriers of the disease, which can aid in preventing the spread of the disease. Screening of healthy fish through PCR based methods allows for the identification of asymptomatic individuals, who may act as potential reservoirs of the pathogen, thereby enabling proactive management strategies to mitigate the spread of the disease.

Initially, the end-point PCR assay was designed by Oyamatsu et al. (1997). The assay was only effective in the diagnosis of CEV genogroup IIa, which primarily infects koi (Adamek et al., 2017a; Matras et al., 2017). The nested PCR was also developed in Japan, but later found to be not very reliable for disease detection (Adamek et al., 2016). As a result of the CEV sequencing from koi, a quantitative real-time PCR was established for the detection and quantification of genogroup II affected tissues (Adamek et al., 2016). As a further development, Centre for Environment, Fisheries and Aquaculture Science (CEFAS) has developed improved nested PCR and qPCR protocols using CEV sequences isolated from common carp and koi in the UK and published by Matras et al. (2017). At the University of Veterinary Medicine in Hannover (TiHo), a quantitative (probe) PCR assay for CEV was later developed based on CEV sequences from samples of koi found in Germany (Adamek et al., 2016). Adamek et al. (2017b) found that this assay was somewhat effective for detecting CEV viruses belonging to genogroup IIa and genogroup IIb. Since, introducing SYBRGreen as an intercalating dye to the TiHo quantitative (probe) PCR assay, improved TiHo's shortcomings, resulting significantly higher diagnostic sensitivity in samples infected from all three genogroups I, IIa, and IIb (Adamek et al., 2017a). For the detection and identification of CEV, Soliman and Matbouli (2018) developed and improved quick and precise single and multiplex isothermal diagnostic techniques based on Recombinase Polymerase Amplification (RPA). For the isothermal nucleic acid detection, the RPA approach is more practical, quick (just 50 minutes are needed for one sample), sensitive, and specific. However, this approach merely requires a stable temperature (Soliman & Matbouli,2018).

It is advised to sample gill tissue when analyzing clinically impaired fish using PCR-based techniques because gill tissue from common carp with CEV infection had statistically significant higher viral loads than kidney, spleen, skin, and gut in studies on experimental virus transmission (Swaminathan et al., 2016; Adamek et al., 2017a).

1.1.5. Water temperature effect on CEV

The optimal conditions for the occurrence of carp edema virus disease in koi are established at water temperatures ranging from 15 °C to 25 °C (59–77 °F). The incidence of CEVD in juvenile koi is often reported during the rainy season, which extends from late June to late July under these permissive water temperatures (Oyamatsu et al., 1997; Miyazaki et al., 2005). Nonetheless, the occurrence of CEVD has also been reported at lower temperatures, ranging from 7 °C to 15 °C (44 –59 °F) in Austria (Lewisch et al., 2015). A Genogroup I of CEV has been linked to disease outbreaks in wild common carp during the winter and early spring, when water temperatures are as low as 6 °C to 9 °C (43–48 °F) (Way and Stone, 2013).

1.1.6. Prevention and treatment for CEVD/KSD

The extensive spread of CEV is likely caused by a lack of efficient preventive procedures in international trade. Currently, no therapy is available for CEVD, and the virus proves challenging at the subclinical level for survivors. For diagnostic labs to accurately identify a CEV infection, they must implement the PCR techniques suggested above. Additionally, extra caution should be taken at various stages, such as transportation and the moving of fish from one pond to another, to prevent the weakening of fish's immune system. Isolating potentially infected fish from others through quarantine is deemed as the most reliable approach to prevent the introduction of pathogens into a facility. Therefore, to avoid the onset of CEV, it is recommended that newly purchased fish should be quarantined for a period of at least 30 days. Monitoring water quality parameters, such as pH, ammonia, and nitrite levels, and maintaining good hygiene practices can also contribute to the prevention and management of CEVD. By following these steps, you can help prevent the spread of CEVD and other diseases and maintain healthy fish populations in your pond or aquarium.

According to various studies, immersing CEV-infected carp in 0.5% salted water lowers the mortality rate among the fish (Seno et al., 2003; Miyazaki et al., 2005). The research conducted by Pikula et al. (2021) investigated the pathophysiology of CEV infection in carp and the resulting metabolic disturbance caused by this disease. The findings revealed significant disruptions in electrolyte and acid-base balance, hematology variables, and blood chemistry profile in CEV-infected fish. It has been noted that the salt treatment helped the affected koi carp reverse alterations in plasma osmolality and hemoglobin concentration that were brought on by viral infection, thus restoring them to their physiological levels and reducing methemoglobinemia. However, the CEV does not, however, disappear or become inactive completely from infected fish while using the same treatment (Seno et al., 2003; Stevens et al., 2018).

1.2. Host range of CEVD

Koi (*Cyprinus rubrofuscus)* and common carp (*Cyprinus carpio* L.) are the most susceptible species to CEV known to date. In order to evaluate the potential carrier role of fish species that share the same habitats with carp, an experimental trial was performed by Matras et al. (2019). This experiment used five species of cyprinids as potential vectors: tench (*Tinca tinca*), roach (*Rutilus rutilus*), bleak (*Alburnus alburnus*), crucian carp (*Carassius carassius*) and Prussian carp (*Carassius gibelio*), and European perch (*Perca fluviatilis*) as representative of the family Percidae. The presence of CEV was then detected using real-time PCR of gill and/or skin tissue of each aforementioned fish species. The CEV DNA was detected in those tissues of all used species with neither clinical signs nor mortality. In that study, data was provided concerning the distribution of the carp edema virus in potential vector species' organs and tissues. However, it is still necessary to investigate whether the pathogens are transferred from vectors to naive fish and other fish species maintained alongside common carp as additional fish.

1.3. Susceptibility to CEVD

Genetic relationships between CEV and other poxviruses are not well understood at present. CEV strains in common carp and koi from around the world appear to represent an entirely new species of poxvirus, based on genetic sequencing (Way and Stone, 2013). Several varieties of common carp such as European and Asian carps have emerged as an outcome of natural geographic separation of common carp groups and domestication, providing a wide range of genetic resources. As already has been published, there may be a large variation in susceptibility among strains that have a different genetic background, as was evident in studies focused on evaluation of susceptibility of different common carp strains to experimental infection of cyprinid herpesvirus 3 (CyHV-3) (Shapira et al., 2005; Rakus et al., 2009; Piačková et al., 2013; Adamek et al., 2019). Comparatively to other viral diseases in fish, scant studies have been conducted to evaluate the susceptibility in CEV-infected carp strains. The researchers have exposed different strains of carp (Amur wild carp, Amur sasan, AS; Ropsha scaly carp, Rop; Prerov scaly carp, PS; and koi), having different susceptibility to CEV, genogroups I and IIa (Adamek et al., 2017b). However, the study focused primarily on determining whether carp strains were susceptible to the disease, and only type I interferon responses as the parameter of non-specific immunity were assessed for CEV affected carp strains.

1.4. Resistance to CEVD

Since measures to control and prevent disease outbreaks are limited in the case of fish compared to terrestrial animals, therefore heritable genetic variation in disease resistance should be identified and combined with other desirable production traits as one of the possibilities for how to protect fish stocks (Tadmor-Levi et al., 2017). Disease resistance in fish relies on a variety of interacting mechanisms ranging from the maintenance of epithelial barriers and the mucosal layer to the nonspecific cellular factors such as phagocytosis by macrophages and neutrophils; nonspecific humoral factors such as lysozyme, complement, and transferrin; and specific humoral and cellular immunity. While genetic factors can influence these protective mechanisms at many stages, some mechanisms seem to be of significance for correlations between variation in immune responsiveness and levels of resistance to infection (Wakelin, 1992). For example, the value of immunological parameters to serve as markers for incorporation in a breeding program is highly dependent on their heritability and

genetic correlation with survival (Fjalestad et al., 1993). Improving genetic disease resistance may offer another preventive measure against infectious diseases, because of its prospects of prolonged protection. In fact, Skamene and Pietrangeli (1991) have shown that the ability of a host to resist infection with a wide range of viral, bacterial, and parasitic pathogens is strongly influenced by genetic factors. There are several approaches to study the genetic influences on disease resistance in animals. One example is the long-term research strategy aimed at a full genetic analysis of the host resistance, as proposed by Festing and Blackwell (1988). Moreover, identification and exploitation of genetic variations and mechanisms for disease resistance in fish might offer a sustainable solution (Chevassus and Dorson, 1990; Fjalestad et al., 1993).

When examining CEVs in fish species (common carp, *Cyprinus carpio* and koi, *Cyprinus rubrofuscus*), variations in sensitivity and resistance are observed. The study based on immune-gene responses concerning CEVD was conducted by Adamek et al. (2017b). In their study, different carp strains, including AS, PS, Rop, and koi carp, were exposed to CEV, genogroups I and II, to investigate their susceptibility to infection. Their analysis comprised the expression of type I interferon, interferon-stimulating genes (ISGs) encoding IFNa2 in gills. The significant finding of this study was the downregulation of IFNa2 and ISGs in response to CEV infection in AS, known to be a highly resistant species compared to other strains/species. Such an effect indicates an activation of the adaptive immunity during the monitored time period (day 6 to 11 post-infection) marked by suppression of cellular immunity through a shutdown of interferon expression. Potential development of humoral immunity and antibody production might have been the reason for resistance to CEV infection displayed by the AS compared to the other strains/species. The mentioned study provides further evidence of the superior immune capabilities of Amur wild carp in combating infectious agents.

1.5. Immunological responses of common carp to disease: Review and investigation of susceptibility to CEV infection

The second chapter of the thesis conducts an extensive review of the immune system and cytokine responses of common carp to various diseases. The objective of the study was to consolidate the available literature on the immune responses, particularly cytokine expression, of common carp or their strains in response to different diseases. The analysis focuses on the role of cytokines, which are biologically active substances involved in inflammation, in the response of fish to both pathogenic and non-pathogenic immune challenges. The production of cytokines can result in the manifestation of symptoms and signs of infections and plays a crucial role in the activation of the recruitment of inflammatory cells, which directly contribute to the clearance of infections. The study underscores the significance of cytokines in the immune response of common carp to various diseases.

Note: More details concerning the cytokines studied in common carp in response to important diseases are summarized in published review (Chapter 2).

The third chapter of the thesis represent the evaluation of the immune responses of four various strains of carp with varying susceptibilities to CEV. The objective of the investigation was to determine the underlying mechanisms that regulate the immune system of carp in response to CEV infection. The investigation specifically focused on characterizing the expression of immune genes in carp strains infected with CEV genogroup I and comparing the responses to those against genogroup IIa. The comparison provided a deeper understanding of the immune system of carp and its capacity to counteract CEV disease. The results of this study are anticipated to contribute new knowledge regarding the immune mechanisms of carp fish and their ability to resist CEV disease and may contribute to the development of novel strategies for the management of CEV disease in carp.

1.6. Objectives of the thesis

- The main aims of the thesis are as follows:
- To evaluate the immune responses in carp strains with different susceptibility to carp edema virus disease.
- To determine immune gene expression pattern in different carp strains infected with CEV genogroup I and compared with responses against genogroup IIa.
- To evaluate the selected immune-related gene expressions in the mucosal organs to CEV genogroup IIa infection.

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CHAPTER 2

CYTOKINES STUDIED IN CARP (*CYPRINUS CARPIO* L.) IN RESPONSE TO IMPORTANT DISEASES

Baloch, A.A., Abdelsalam, E.E.E., Piačková, V., 2022. Cytokines studied in carp (*Cyprinus carpio* L.) in response to important disease. Fishes 7, 1, 3.

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Review

Cytokines Studied in Carp (*Cyprinus carpio* L.) in Response to Important Diseases

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Abstract: Cytokines belong to the most widely studied group of intracellular molecules involved in the function of the immune system. Their secretion is induced by various infectious stimuli. Cytokine release by host cells has been extensively used as a powerful tool for studying immune reactions in the early stages of viral and bacterial infections. Recently, research attention has shifted to the investigation of cytokine responses using mRNA expression, an essential mechanism related to pathogenic and nonpathogenic-immune stimulants in fish. This review represents the current knowledge of cytokine responses to infectious diseases in the common carp (*Cyprinus carpio* L.). Given the paucity of literature on cytokine responses to many infections in carp, only select viral diseases, such as koi herpesvirus disease (KHVD), spring viremia of carp (SVC), and carp edema virus disease (CEVD), are discussed. *Aeromonas hydrophila* is one of the most studied bacterial pathogens associated with cytokine responses in common carp. Therefore, the cytokine-based immunoreactivity raised by this specific bacterial pathogen is also highlighted in this review.

Keywords: common carp; immune response; koi herpesvirus (CyHV-3); carp edema virus (CEV); spring viremia of carp (SVC); *Aeromonas hydrophila*

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1. Introduction

The common carp (*Cyprinus carpio* L.) is one of the most important cultivated fish species contributing to the global economy. Carp are currently cultured worldwide at a rate of over 3 million metric tons/year, which represents around 10% of the global production of freshwater fish in aquaculture [1,2]. Historically, the common carp was cultured only for human consumption, beginning around 3000 years ago in China (FAO, 2006). Currently, it is a widely cultured fish species in over 100 countries, and not only used for human consumption but also as an ornamental fish and for recreational fishing purposes [2]. In recent decades, the common carp has been subjected to intensive rearing patterns, which may induce stress and lead to a weakened immune system, increasing susceptibility to various pathogens [3,4].

Thanks to more advanced approaches and different, newer technologies, we now have a deeper understanding of the immune processes protecting fish against the effects of infectious agents, which can be tracked to help prevent diseases.

Great progress has been made in isolating and characterizing cytokines in fish in recent years. However, immunoreactive responses vary due to the presence of different pathogenic stimuli, which is a focal issue for research. Evaluating cytokine responses, which develop in the early stages of infections, is essential in determining the behavior of both host and pathogen during and/or after the infection. Consequently, preventive measures, such as the development of vaccines and protective approaches related to the fishes' environment, can be carried out. In this review, particular attention has been paid to discussions of the cytokine responses provoked in common carp due to cyprinid herpesvirus 3 (CyHV-3), spring viremia of carp virus, carp edema virus, and Aeromonas hydrophila infections. The

selected disease-causing agents represent important pathogens that occasionally infect farmed carp worldwide, resulting in substantial economic losses due to high mortality rates and/or officially ordered culling of fish stocks, as in the case of KHV.

Koi herpesvirus disease (KHVD) was first diagnosed in the late 1990s in Israel, and it has since spread across the world, primarily due to the ornamental fish trading industry [1]. Due to its infectivity, lethality, and economic impact, it was listed as a non-exotic disease in a European Council directive (2006/88/EC), and more recently in Annex II of Animal Health Law (Regulation (EU) 2016/429). It is also an OIE (World Organization for Animal Health) listed disease [2,3].

Carp edema virus disease (CEVD) first occurred in European fish farms about 10 years ago. Clinical signs are very similar to KHV (gill necroses, sunken eyes, irregular skin-mucus production, mortality). The disease often appears in lower water temperatures than KHVD [4]. The causative agent is probably the same virus responsible for "sleepy koi disease", diagnosed in the 1970s in koi farms in Japan [5]. However, several different genogroups of this viral pathogen have recently been distinguished by molecular biology tools [6]. Although the spread and economic impact of CEVD are similar to KHVD, to date, it remains a non-notifiable disease, and its prevalence is relatively unknown. In the OIE, it is listed as an emerging disease.

Unlike KHV and CEV, which are host-specific only for carp and koi, SVCV has been diagnosed in most members of the *Cyprinidae* family, as well as in several other fish species [7]. Although the disease has not caused significant economic losses in carp farming in recent decades, it is still one of the diseases monitored by the OIE. The virus is present in most European countries, North America, South America, and Asia (OIE, 2017); however, owing to the absence of systematic monitoring, information about the true spread of SVCV worldwide is not available.

Aeromonas hydrophila belongs to the motile aeromonads group, which are the most abundant bacteria in aquatic environments. It is responsible for causing carp erythrodermatitis; sometimes, the disease is recognized as "motile *Aeromonas* septicemia" (MAS) [8]. An opportunistic pathogen, *A. hydrophila* usually causes disease in fish under stress in various fish species [9]. The conditions caused by *A. hydrophila* infection in fish farms are accompanied by high death rates and, subsequently, large economic losses [10].

2. Fish Immune System

The immune system of vertebrates consists of several immune organs, which can be divided into two categories, primary and secondary. Within these organs, immune cells, including differentiated and non-differentiated leukocytes, represent an essential part of the lymphoid system. In addition, they can be disseminated in blood or other tissues, along with various substances produced by these cells. The immune organs of teleost fish are somewhat different from those of other vertebrates, with fewer consistent lymphatic compartments distinguishing the lymphoid system from the circulatory system. However, the tissues of lymphatic organs are equally responsible for the production and storage of lymphocytes and providing sites for their interactions with antigens [11]. The primary lymphoid organs in fish include the head kidney and thymus, while the secondary lymphoid organs consist of the spleen, trunk kidney, and mucosa-associated lymphoid tissue (MALT), such as gills, skin, intestine, oral and nasal mucosa, and urogenital tract [12,13]. According to anatomical location, MALT can be further subdivided into skin-associated lymphoid tissue (SALT), gut-associated lymphoid tissue (GALT), and gill-associated lymphoid tissue (GIALT) [14,15].

The teleost immune system is similar to that of higher vertebrates, comprising both specific (adaptive) and non-specific (innate) responses to overcome invasion by various pathogens, such as viruses, bacteria, and parasites [16]. The non-specific immune response is considered the very first defense against pathogens. Unlike other vertebrates, fish primarily depend on the non-specific immune system for survival during early embryonic development [17]. Not only that, but due to fish being poikilothermic, they lack some of the

conventional characteristics of adaptive immunity represented by limitation in antibody defenses and slow proliferation, maturation, and memory possessiveness of lymphocytes. Therefore, in fish, innate immune responses are considered fundamentally important defenses and are mainly classified into three basic mechanisms: physical (epithelial/mucosal) barrier, cellular component, and humoral responses [18]. The mucosal barrier of the skin, gills, and gut comes into direct contact with causative agents of diseases found in the environment. Hence, it constrains the invading pathogens through a series of mechanisms involving the secretion of antimicrobial humoral factors by immune cells or tissues. These factors may include specific peptides that are able to identify certain pathogen-associated molecular patterns (PAMPs), such as microbial endotoxins such as lipopolysaccharides (LPS), and polysaccharides, or viral DNA and RNA, bacterial DNA, or other molecules that are naturally found on the surface of different microorganisms [19]. Such antimicrobial agents are known as immunological identifiers due to their ability to recognize molecules on pathogens' surfaces and aid in eliminating them either through direct destruction by phagocytosis or via regulation of cellular immune responses. Examples of these identifiers include members of the major histocompatibility complex (MHC), lytic enzymes, growth inhibitors, antibodies, antibacterial peptides, chemokines, and cytokines.

3. Cytokines

Cytokines are proteins produced and secreted by a variety of immune cells (T and B lymphocytes, mast cells, and endothelial cells) with development, differentiation, and activation capabilities that influence the nature of immune responses [20]. Cytokines are engaged in various immune reactions, from induction of non-specific immune response to the generation of the primary effector cells of the innate immune system known as cytotoxic T cells, up to the production of antibodies by B cells [21]. The cytokines can be grouped into distinct families based on their structural characteristics, such as interferons (IFNs), interleukins (ILs), tumor necrosis factors (TNFs), transforming growth factors (TGFs), and chemokines [22].

Interferons (IFN) play a crucial role in inhibiting viral replication in different viral infections in vertebrates [23]. The underlying mechanism involves recognizing and inhibiting viral nucleic acid via binding to specific cell receptors and initiation of gene expression cascade responsible for encoding antiviral proteins [24]. For instance, an experimental study on infectious pancreatic necrosis virus in Atlantic salmon evidenced that AS-IFN can specifically inhibit virus activity following the induction of interferon-stimulated genes [25]. However, unlike mammals, where interferons are divided into three classes (type I, II or IFNγ, and III), in fish, only classes I and II have been discovered [26,27]. Nevertheless, fish seem to have a collection of cytokines akin to those of mammals [28]. Various homologs of the cytokines and their suppressors reported in mammals were successfully cloned in several fish species, either purified and identified as native fish proteins or theoretically predicted according to a similarity in structure or sequence [29].

In addition to interferons, interleukins (IL) are also important cytokines in the fish immune system, produced by different types of leucocytes [30]. The most important interleukins consist of pro-inflammatory (IL-1, IL-2, IL-6, IL-11, IL-17, IL-18, and IL-33) and anti-inflammatory (IL-4, IL-6, IL-10, and IL-13) cytokines. The mechanistic effects of these molecules tend to either suppress the activities of individual members or induce pro-inflammatory effects [31]. For instance, IL-10 can be expressed by all immune system cells, including dendritic cells (DC), macrophages, mast cells, natural killer cells (NK), eosinophils, neutrophils, B cells, and $CD8^+$ T cells. Despite being an anti-inflammatory cytokine, IL-10 produced by these cells seems to inhibit the production of proinflammatory cytokines in most cases. Hence, it might be viewed as a pleiotropic cytokine with both robust immunosuppressive and anti-inflammatory capabilities [32]. Among several cytokines, IL-1 was found to be the earliest cytokine identified in fish [33]. In mammals, genes encoding IL-1α and IL-1β are recognized, whereas, in fish, only genes encoding IL-1β have been discovered as of yet, which is homologous to that of mammals [29]. Furthermore, in

fish, the role of interleukins represented mainly by IL-1β is proposed as analogous to that in mammals through regulation of immune responses via the early activation of T cells [34].

Tumor necrosis factor (TNF) is a critical cytokine that plays an essential role in physiological and pathological processes. It consists of various family members, including TNF alpha (TNF-α) and TNF beta (TNF-β). Both are expressed in macrophages, T, B, and natural killer (NK) cells, and in the TNF-related apoptosis-inducing ligand and CD40 ligand (primarily expressed on the surface of T cells). Using different mechanisms of action than interferons and interleukins, tumor necrosis factors α and β are confirmed to promote phagocytosis and nitric oxide production in teleost during viral and bacterial infections [35,36]. Both TNF family members (TNF-α and TNF-β) are produced from macrophages and T lymphocytes, respectively, while also being expressed by other immune cells at low levels. In fish, TNF-α has been cloned in several species, including common carp (*C. carpio*) [37], gilthead seabream (*Sparus auratus*) [38], catfish (*Ictalurus punctatus*) [39], grass carp (*Ctenopharyngodon idella*) [40], zebrafish (*Danio rerio*) [41], and sea bass (*Dicentrarchus labrax*) [42]. In light of its importance for the mammal immune system, it is surprising that no TNF-β gene has been identified in fish. However, the missing function of TNF-β may be performed by alternative proteins. Analyses of phylogenetic trees and genetic co-localization in certain loci of fish chromosomes in some teleost indicate the existence of two types of TNF-α, named I and II. Type-II TNF-α, which is reported in catfish (type-II catfish TNF-α) [39] and trout (type-II trout TNF-α3) [38], proposedly have a similar function to that of TNF-β in mammalian species.

Transforming growth factor β (TGF-β) is a key inhibitory and inflammatory cytokine responsible for tissue repair during pathophysiological reactions [43]. TGF-β regulates cell development, differentiation, and proliferation and modulates different leukocytes and their lineages, including lymphocytes, macrophages, and NK cells [44]. In mammals, TGF-β maintains immune tolerance and is a well-known immunosuppressive cytokine [45]. The immunosuppressive effect is primarily due to the stimulation of T lymphocytes. For instance, during in-vitro study, the activation of resting T cells limits the lymphocytes' clonal expansion, resulting from increased levels of TGF-β receptors and their mRNA expression [46]. In teleost immunity, the role of TGF-β appears to be parallel to that in mammals. Although limited investigations of TGF-β immune activity in fish have been carried out, a few studies have examined its role in some fish species. For instance, in a study on the top mouth culter (*Culter alburnus*), TGF-β1 was found to express highly during lipopolysaccharides (LPS) stimulation, where it blocked the mRNA expression of pro-inflammation cytokines, such as IL-β and TNF-α [47]. A similar immune suppressive function of TGF-β was noticed in LPS treated common carp and goldfish when the nitric oxide response of TNF-α activated macrophages were significantly reduced, and considerable downregulation of TNF-α was noticed [48,49].

Chemokines, also known as chemoattractant cytokines, regulate the immune cells and control their migration to the site of inflammation. In mammals, chemokines are divided into four subfamilies according to their peptide structures: CXC (α), CC (β), C, and CX3C classes [22]. Fish chemokines (CXCα, CXCβ1, CCβ, and C) and their receptors are described in some teleost, including zebrafish, rainbow trout, carp, catfish, gilthead seabream, and Japanese flounder [50]. Limited progress has been made in studying their role in teleost immunity. Nevertheless, their contribution to immunity in several vertebrates through localizing and regulating the function of immune cells calls for more investigation about their importance in fish [50].

3.1. Cytokine Studied in Response to Cyprinid Herpesvirus 3 Infection

Cyprinid herpesvirus 3 (CyHV-3), also known as koi herpes virus (KHV), is the causative agent of koi herpesvirus disease (KHVD). This DNA virus belongs to the family *Alloherpesviridae* [51]. The disease affects both common carp (*C. carpio*) and its ornamental variety, koi (*Cyprinus rubrofuscus*). External clinical signs include lethargy and anorexia followed by excessive mucous production on the gills and skin and necrosis of gill tissue.

Petechial bleeding spots can also be seen in the final stages of infection on the trunk, vent, and around the mouth [52,53]. KHV is categorized as an emerging disease that causes massive mortality resulting in substantial economic losses to the aquaculture industry (FAO, 2010).

The coevolution of fish herpesviruses and their hosts is believed to have occurred about 400 to 450 million years ago [54,55]. Over time, hosts have adapted a more developed and divergent immune system to safeguard themselves in the face of viral or other pathogenic infections. In different vertebrates, including humans, it has been proven that the non-specific immune reaction against herpesviruses generally incorporates an activation of (NK) cells and the production of interferons and different types of interleukins [56].

Interferon (IFN) plays a crucial role in innate immunity to guard the body against viral invasion. This cytokine significantly contributes to the early containment of herpesvirus infections because of its immunoreactive properties. Several type 1 IFN reactions, including interferon regulatory genes (ISGs), are characterized during viral infections [57,58]. Multiple studies have also shown that recombinant interferon or the stimulation of type I IFN production has a protective effect against numerous fish viruses, whether performed in vitro or in vivo. The magnitude of type I IFN responses are essential for increased resistance to virus-induced mortality in fish infected with viruses with more complex genomes, like alloherpesviruses. However, in the case of CyHV-3 infection, skin is considered a major entry organ and the primary site for infection in carp [59,60]. Fish skin serves as a good protection barrier from different types of pathogens [61]. Few studies have been carried out to gain insight into the type I IFN response during CyHV-3 infection exclusively. For instance, a relationship between the upregulation of type 1 IFN response and CyHV-3 infection was observed in the skin of infected common carp [57] (Table 1). However, the localized intervention of IFN-1 on the skin interprets the absence of interferon type 1 response during in-vitro testing on *C. carpio* brain (CCB) cells infected with CyHV-3 [62]. Moreover, lack of type 1 interferon response on other sites could be extrapolated as antiviral capabilities adopted by koi herpesvirus against IFN-1.

Table 1. List of cytokines studied during cyprinid herpesvirus 3 (CyHV-3), spring viremia of carp virus (SVCV), carp edema virus (CEV), and *Aeromonas hydrophila* (*A. hydrophila*) infections in common carp (*C. carpio*).

CyHV-3				
Assay Condition	**Tissue/Cell Type**	**Studied Cytokine**	**Observed Effect**	**Reference**
in vivo	kidney	IFNαβ, IL-12p35	↑	[63]
	gill	type 1 IFN, Vip, PKR	↑	[64]
	gill, kidney, spleen	IL-10	↓	[65]
	spleen	IL-1β, IL-10, IL-12p35, IL-6 and IFNαβ	↑	[66]
	skin, head kidney	Type 1 IFNs	↑	[67]
	anterior kidney	IL-10	↑	[68]
	spleen	Type 1 IFN, Vip2 and IL-8	↑	[69]
	spleen	IFNγ-1, IFNγ-2, IL-1β, IL-10, and IL-12	↑	[70]
	spleen, kidney, intestine	IL-10	↑	[71]
	spleen	IFNγ-2, IL-6, Mx and IL-8	↑	[72]
	gills	IL-10 and TNF	—	[73]
	muscles	IL-1β, TNF-α, IFNγ2ab, ISGs (Mx1, Vip2 and PKR3)	↑	[74]
	serum	IL-1β	—	[75]
in vitro	head kidney leucocytes	type 1 IFNs	↑	[62]
in vitro/in vivo	gill, kidney, head kidney, skin	type 1 IFN	↑	[76]
	plasma	TNF-α	↑	[77]
	spleen	IL-10	↓	[78]

Table 1. *Cont.*

Assay Condition	Tissue/Cell Type	Studied Cytokine	Observed Effect	Reference
CyHV-3				
svcv				
in vivo	spleen, head kidney, intestine, thymus, blood	IRFs	↑	[79]
in vitro	head kidney leucocytes (HKLs)	type 1 IFNs	↑	[62]
in vitro/in vivo	kidney and spleen	type 1 IFN, Mx1, Rig1	↑	[80]
	skin, kidney, head kidney	IFNα2 and Vig1	↑	[76]
	muscles and blood	IL-1β, IL-6, TNF-α, IFNγ2a, IFNγ2b, IFNø1, IFNø2, Mx1, Mx2, Vip 2, PKR3	↑	[81]
CEV				
in vivo	gill	Type 1 IFN (Vip and PKR)	↑	[64]
A. hydrophila				
in vivo	spleen	IL-1β, IL-10, IL-12, IL-6, IL-8, IRFs (1, 4, 7 and 8)	↑	[82]
	head kidney	IL-1β, IL-10, TNF-α, Cc and Cxc-chemokines	↑	[83]
	spleen and head kidney	IL-1β, TNF-α	—	[84]
	spleen and liver	IL-1β, IL-10	↑	[85]
	spleen, kidney, and liver	IL-17	↑	[86]
	head kidney and pituitary	IL-1β	↑	[87]
	head kidney, spleen, foregut, hindgut, and endothelial progenitor cells (EPCs)	IRF9, Type 1 IFN, PKR, ISG15, TNF-α	↑	[88]
in vitro	spleen, head kidney, foregut, hindgut, peripheral blood leucocytes (PBLs) and head kidney leucocytes (HKLs)	IRF10	↑	[89]
in vitro/in vivo	head kidney leucocytes (HKLs)	IL-1β, IL-10	↑	[90]
	head kidney and trunk kidney	IL-17, IL-1β	↑	[91]

IFN—interferon; IL—interleukin; TNF—tumor necrosis factor; TGF—tumor growth factor; Mx—myxovirus resistance protein; Vip—viperin; PKR—protein kinase RNA-activated; IRF—interferon regulatory factor; Vig-1—VHSV-induced gene; Rig-1—retinoic acid-inducible gene-1; ↑—overall upregulation; ↓—overall downregulation; — —no changes.

During infection with herpesviruses, activation of adaptive immune response occurs in the later stages. The response is carried out by stimulation of the natural killer T cells and B lymphocytes to induce interleukins, in addition to TNF-β and IFN-γ, to initiate and prolong the responses by enhancing the production of antibodies [92]. However, the role of these specific cytokines is not fully elucidated in CyHV-3 infection. Rather, infected carp produce specific immunoglobulins (Ig) and mount cell-mediated immune responses [93]. Fish that survive a CyHV-3 infection acquire resistance, leading to a remarkable reduction in mortality [94,95]. Despite that, the capacity to build up a long-lasting latent infection is the hallmark of all known herpesviruses, including fish herpesviruses. Those latent infections are either controlled through the virus mimicking host immunoreactions or by encoding their own antiviral-like proteins such as IL-10 [96–98].

In order to replicate more effectively, several viruses encode their own viral IL-10 homolog (vIL-10), which exert an immunosuppressive effect in the host at the beginning of the infection [96,97]. CyHV-3 vIL-10 was initially described in common carp and the European eel [98]. Later on, Sunarto et al. [65] also reported that CyHV-3 captures an IL-10 gene from the host and modulates its features to tackle the host immune response. However, an in-vitro study conducted by Ouyang et al. [78] demonstrated that CyHV-3 ORF134 (the gene encoding an IL-10 homolog) was neither essential for viral replication nor virulence in common carp.

Nevertheless, CyHV-3 appears to manipulate host antiviral mechanisms more prominently via interaction with the TNF-α pathway. A study conducted by Rakus et al. [77] revealed a significant connection between CyHV-3 infection and the development of inflammatory responses controlled by TNF-α. Based on the findings of the study, infected

carp demonstrated a delay in fever manifestation, which is an essential infection phase that promotes viral replication. This modulation in disease development was associated with viral CyHV-3 ORF12 encoding the TNF-α soluble decoy receptor, which is known to block cytokine activity [77].

The research of gene expression in carp has yielded an insightful perception of the CyHV-3 pathogenesis associated with cytokine secretion. Specific groups of cytokines including IFNγ-1, IFNγ-2, IL-12, IL-10, IL-1β, TNF-α1, and genes of major histocompatibility complex (MHC-II), are found to be over- or under-expressed, respectively, to the ongoing infection stage [70]. For instance, fish tested during acute phases of infection show a higher expression rate in all interleukin and interferon genes, while class II MHC and TNF-α1 genes are downregulated [70]. Furthermore, a survey conducted by Rakus et al. [66] on two carp lines (R3 and K) following CyHV-3 infection has revealed a disparity in the kinetics of cytokine genes expression on different days post-infection. The R3 line exhibited significant upregulation of IL-1β, IL-10, IL-12, and MHC class I genes and more disease resistance compared to the K line, whereas no significant differences in IFN expression were detected in both lines during the infection. It can be speculated that differential expression of those cytokine genes in carp lines could be due to host-related genetic factors incorporating the progress of the infection phase and shifting to adaptive immunity.

The use of cytokine or cytokine inducing cells (i.e., macrophages, B cells, and dendritic cells) as an adjuvant to improve the immunogenicity of DNA vaccines in fish is another growing research interest [99,100]. A potential protective effect of IL-β in combination with the CyHV-3 ORF25 DNA vaccine has been observed in common carp [75]. Likewise, the mammalian granulocyte–macrophage colony-stimulating factor, which in mammals is described as a regulator cytokine of immune cells proliferation, differentiation, and maturation [101], has also improved the immunogenicity of DNA and subunit vaccines in fish [102]. Moreover, the effect of the CyHV-3 ORF25 DNA vaccine on IL-β, cxca, cxcb1 chemokines, and interferon-stimulated genes (Mx1, vip2, pkr3, and isg15) have been examined in muscles of common carp by Embregts et al. [74]. In their study, they used ORF25-based DNA vaccine in different vaccination routes, i.e., intramuscular injection (i.m.) or oral gavage, in one or multiple doses. A strong immunostimulant effect was observed through the repeated i.m. injection, which resulted in cytokine expression and interferon-stimulated genes.

3.2. Cytokine Studied in Response to Spring Viremia of Carp Infections

Spring viremia of carp (SVC) is a highly contagious viral disease affecting carp (*C. carpio*) and other cyprinid species. The disease is caused by the *Carp sprivivirus* (originally SVC virus), a single-stranded RNA virus belonging to the family *Rhabdovividae* [7,103]. The condition is associated with edematous symptoms such as exophthalmia, edema of the underlying tissues, and abdominal distention [7]. Gross signs include petechial hemorrhages on the eyes, skin, gills, and internal organs, specifically on the swim bladder [104].

The SVCV genome encodes five different viral proteins, including matrix protein (M), nucleoprotein (N), glycoprotein (G), phosphoprotein (P), and viral RNA-dependent polymerase, important for viral transcription and replication, endocytosis, and infectivity [105]. The accessibility of the entire genomic information of SVCV, and its homology to mammalian rhabdoviruses, has allowed us to understand the function of these proteins more accurately [7,106].

Rhabdovirus infections, in fish, are controlled through the responses of group I and II IFNs. The recombinant IFNs group I appears to inhibit virus replication at any of the replicative stages [107–110]. The mechanism of rhabdovirus containment by group II IFNs varies intrinsically [110]. Recombinant IFNs-II was found to induce a protective effect against SVCV infection in zebrafish through upregulation of interferon regulatory genes such as myxovirus resistance protein (Mx) and viperin. On the contrary, group 1 IFNs upregulate both ISGs and pro-inflammatory cytokines (IL-10β and TNF-α) more persistently [111]. Conclusions from in-vitro and in-vivo research in carp indicate alternative responses of

IFNs-I during SVCV and CyHV-3 infections. For instance, in a study on CCB cells infected with either SVCV or CyHV-3, a higher IFN-I upregulating response was detected through SVCV compared to CyHV-3, which oppositely diminished IFN-I production [62]. A similar trend of IFN response was noticed when different common carp strains (Amur wild carp, Amur sasan, AS; Ropsha scaly carp, Rop; Prerov scaly carp, PS; and koi) were challenged with the same viruses [76]. Rop was found to be more resistant to SVCV infection than the PS strain. During CyHV-3 infection, both Rop and AS displayed an increased survival rate compared with the PS strain. Interestingly, these observations were associated with increased activity of IFN-I among the SVCV resistant carp more than that in the CyHV-3 resistant group. The contribution of type 1 interferon to prevent SVCV, but not CyHV-3, infection appears to be a host–pathogen-specific interaction with the SVC virus.

Type 1 interferon, in addition to IL-12, are collectively known as signal-3 cytokines, which have been shown to activate clusters of differentiation 8 (CD8) T cells [112]. The cluster comprises CD8a and CD8b; both are dimeric co-receptors recognizing peptides presented by MHC class I molecules. They have a crucial role in immune defenses against various pathogens, including viruses. Both type 1 IFN and IL-12 were found to support the expansion and differentiation of CD8 T cells in vitro [113,114]. CD8 T cells also display an equivalent gene expression profile in the presence of either type 1 interferon or IL-12 and also upon in vitro stimulation. A survey conducted by Forlenza et al. [93] evaluated transcription of signal-3 cytokines, evident a concomitant of IL-12 and type 1 interferon, with CD8ab during SVCV infection in common carp. Precisely, signal-3 cytokines coincided with CD8ab upregulation at day 4 post SVCV infection. Initiation of CD8ab via signal-3 cytokines during SVCV infection indicates that these pro-inflammatory molecules play a regulatory and reciprocal role via the MHC-I pathway, which involves activating antigen-directed adaptive immunity.

Earlier research revealed the presence of active IFNs in sera of fish infected with viral hemorrhagic septicemia virus (VHSV), infectious hematopoietic necrosis virus (IHNV), and SVCV [115]. More recent follow-up research has confirmed the presence of these similar interferons via transcriptional upregulation of genes after experimental infection with the viruses mentioned above [111,116].

IFN-γ, in fish, generally evokes an immune response that features overriding virus-replicative activity. For instance, recombinant IFN-γ upregulates some interferon regulatory genes, including ISG12, ISG15, and Mx [117], and shows antiviral effects, such as the inhibition of viral structural proteins synthesis and the reduction of virus titer in different cell lines of Atlantic salmon [118]. In cyprinids, i.e., zebrafish, the biological activities of IFN-γ, in addition to groups 1 and 11 of IFNs that are classified as type I interferons in fish, have been evaluated in vivo during SVCV infection. Strikingly, and unlike groups 1 and 11 IFN-I, zebrafish interferon (zfIFNγ) failed to produce pro-inflammatory genes and antiviral effects when administered [111].

As previously mentioned, IFNs have a significant role in establishing an antiviral response against *Carp sprivivirus* involving initialization of the adaptive immunity. Despite that, *Carp sprivivirus* was reported to have the ability to evade host immune response [119]. Studies conducted by Li et al. [120] and Lu et al. [121] demonstrated that the overexpression of SVCV phosphoprotein N and glycoprotein G inhibits IFN synthesis in carp and zebrafish by reducing IFN transcription in host cells.

Interferons are not the only cytokines involved in the immune response during *Carp sprivivirus* infection. Some studies also observed an interleukin activity in response to this pathogen. For instance, carp IL-10 paralogues IL-10a and IL-10b possess similar protein sequences and phagocytes-provoking bioactivity were tested on carp head kidney cells. IL-10b had a low tissue constitutive expression in the liver, gut, gills, spleen, thymus, peripheral blood leucocytes, head, and trunk kidney when assessed in the absence of pathogens. However, it was upregulated during SVCV infection in the head and trunk kidney, followed by other tissues. Contrary to this, IL-10a gene expression did not change

throughout the infection period despite its tissue constitutive expression being higher in healthy tissue [122].

3.3. Cytokine Studied in Response to Carp Edema Virus Infection

Carp edema virus disease (CEVD) or sleepy koi disease (KSD) is a rising threat to koi and carp aquaculture. The disease was first reported in koi carp farms in Japan in 1974, where it caused substantial mortalities [123–125] and economic losses. The nomenclature of sleepy koi disease is attributed to the behavioral abnormalities showed by affected carp, including lethargy and unresponsiveness. Consequently, the disease is called sleepy koi, and fish can be seen lying in the bottom of tanks for extended periods [126]. Gross lesions incorporating spreading hemorrhagic skin lesions with edema, particularly in the abdomen, pale gills, sunken eyes, and ulcerative inflammation on the anus may also be seen [127–129]. Overproduction of mucus on the skin and gills is also observed [130,131].

Carp edema virus (CEV), belonging to the *Poxviridae* family, consists of double-stranded DNA about 250–280 nm in diameter, with a mulberry-like structure [128]. Gills seem to be the primary site for the infection. In diseased fish, poxvirus-like structures were confirmed by electron microscopy in morphologically altered gill tissue [125,126,132]. Three different genogroups of CEV have been so far characterized: I, IIa, and IIb [5,6]. Genogroup I, which has been detected in most European waters, is mainly infective to common carp. Genogroup IIa is almost entirely, but not exclusively, reported in koi, while genogroup IIb has been detected in both carp and koi samples.

Poxviruses are contained through innate immunity undertaken by the inflammatory and NK cells [133]. These responses control viral replication and mount an antigenic adaptive response [34]. Similar to other viral infections, interferons (α, β, and γ) play an important role during poxvirus infections. Interferons produced by NK cells, fibroblasts, lymphocytes, and leucocytes conclusively control poxvirus replication. Nevertheless, poxviruses also modulate the host immune responses to guarantee their survival [134]. The implicated mechanism incorporates interruption of ISGs synthesis resulting in IFN inhibition. Furthermore, poxviruses possess immunomodulating genes able to competitively antagonize the IFN-α via encoding a homolog of the ligand-binding receptors chain for this interferon [135].

However, during CEV infection in carp, the host–virus interaction is reciprocally impacted by host genetic competitiveness and virus genomic characteristics. In other words, the carp edema virus, although it still remains unclassified, can probably vary in its genotype, as several emerging genogroups have so far been identified among carp populations worldwide. On the other hand, these genogroups are seemingly selective mutations targeted towards the numerous variants within the common carp species. It is reported that the underlying strains of carp highly influence the expression profile of cytokines during infections, specifically the IFN and ISGs [69].

The only study based on IFN and ISGs concerning CEVD was conducted by Adamek et al. [64]. In their study, different carp strains, including AS, PS, Rop, and koi carp, were exposed to CEV, genogroups I and II, to investigate their susceptibility to infection. Their analysis comprised the expression of type I interferon, ISGs encoding IFNa2 in gills. Amur wild carp was found to be more resistant to the infections than the other three strains/species. The significant finding of this study was the downregulation of IFNa2 and ISGs in response to CEV infection in AS, known to be a highly resistant species compared to other strains/species. Such an effect indicates an activation of the adaptive immunity at the proposed time of infection marked by suppression of cellular immunity through a shutdown of interferon expression. Potential development of humoral immunity and antibody production might have been the reason for resistance to CEV infection displayed by the AS compared to the other strains/species. Likewise, a lack or delay in initiating an antigenic-based adaptive immunity in susceptible strains could be responsible for the high mortalities. A similar cytokine cellular immune-responsiveness trend is reported in association with CyHV-3 and SVCV. Previous transcriptomic analysis of differentially

expressed immune genes during CyHV-3 infection elucidated a rapid upregulation of IFNs and ISGs in susceptible strains versus resistant ones [69]. These observations were supported by other findings reporting an influence of carp strain on the interferon expression rate in fish infected with CyHV-3 or SVCV [76]. In contrast to CyHV-3 and SVCV infections, where the mechanism of IFN response has been studied quite thoroughly, there remains a paucity of research on IFN response during CEV infection in common carp. Overall, the response of IFNs in different carp strains/species to CEV infections is consistent with previous studies [69,76]. However, more research is required to support the IFNs response to CEV infection in carp strains.

3.4. Cytokine Studied in Response to Aeromonas hydrophila Infections

Aeromonas hydrophila is a rod-shaped Gram-negative bacterium and one of the most common bacterial pathogens infecting fish in freshwater [136]. *Aeromonas hydrophila* naturally inhabits the gastrointestinal tract of fish [83]. This bacterium predominantly affects teleosts that are highly susceptible to the infection, such as common carp, catfish, goldfish, and other tropical and ornamental fish [137,138]. Infected fish manifest different clinical signs, including swimming abnormalities, generalized edema, pale gills, and deep dermal ulcers [139]. In common carp, a distended abdomen and scale blisters on the skin are the prominent signs of this disease [140].

Eliminating bacterial pathogens in the host is carried out through an immediate and non-specific response. Upon the stimuli of the immune system with bacterial agents, the recruitment of several phagocytes involved in cytokine production like neutrophils, macrophages, and dendritic cells takes place [141]. During *A. hydrophila* infections, the secretion of cytokines such as ILs, IFNs, and TNFs hold great importance. Interleukins seem to be a focal point for immunological research interest, followed by tumor necrosis factors and interferons.

It is believed that the expression of IL1-β, IL-10, and IFN in carp during *A. hydrophila* invasion is triggered primarily by the toll-like receptor 18 (Tlr18), as indicated by a study on the epithelioma papulosum cyprini cell lines (EPC) [90]. This important pattern recognition molecule usually exists on macrophages and dendritic cells and has a fish-specific expansion that plays a crucial role in innate immunity against pathogens via cytokines activation.

In response to bacterial infections, IL-1β induces adhesion molecules (selectins, integrins, and cadherins), which assist in recruiting neutrophils to the site of inflammation [142]. Several studies have been conducted to evaluate the response of IL-1β during *A. hydrophila* infections. For instance, carp subjected to high temperature (30 °C) for 30 days and subsequently challenged with *A. hydrophila* had significantly elevated IL-1β and TNFα expression in the spleen and head kidney. Other immune or stress-related genes, such as inducible nitric oxide synthase (iNOS), glucocorticoid receptor (GR), and superoxide dismutase (SOD), were also suppressed [84].

The emergence of *A. hydrophila* in the carp population is common around the globe, but using antibiotics as a preventive measure has been questioned due to bacterial resistance and potential risk to consumer health [143]. Additionally, *A. hydrophila* has a significant strain diversity with plentiful pervasiveness in aquatic environments [144]. Therefore, developing an effective vaccine to control this pathogen remains an issue of interest. At present, various *A. hydrophila* vaccines have been developed, such as inoculations comprising killed whole bacteria, components of the pathogen macromolecules, biofilms, or non-replicated protein isolates [34,145–149]. Most of these vaccines, however, exhibit non-functional immunogenicity. Considering this fact, multiple research attempts have aimed to evaluate the efficiency of live, attenuated (XX1LA) or killed (by formalin) *A. hydrophila* to induce an early and adequate immune stimulation in carp. In both cases, upregulation of the IL-1β- and IL-10-related genes occurs in the spleen and liver specifically, as indicated in a study conducted by Jiang et al. [85]. Although other parameters, such as survival rate, antibody titer, and serum lysozyme were vaccine-dependent, it can be demonstrated

that the IL-1β and IL-10 are major game changers in the innate immune defense against *A. hydrophila* with association to tissue-specific gene regulation.

Interleukin IL-17 is another nominated key pro-inflammatory cytokine involved in immunoreactions against bacterial infections in carp. In fact, IL-17 with pro-inflammatory characteristics plays an essential role against a wide range of viral, bacterial, and fungal infections [150]. Providing mucosal immunity at the first entry sites, i.e., gills and skin, against various pathogens is the hallmark of this cytokine, which exists abundantly in mucosal and immune tissues [151]. In mammals, interleukin comprises seven ligands, from IL-17A to IL-17F. IL-17A and IL-17F are pivotal cytokines that play a significant role in autoimmune disorders and immunological responses to pathogens [152]. These cytokines regulate gut microbiota and act as the first line of defense in mucosal tissue against invading pathogens.

In fish, all seven ligands have been identified, and transcriptional studies have shown the tissue-specific ligand role of IL-17 in all tissues [151]. Unsurprisingly, these ligands' expression was abundant in mucosal tissues, signifying their essential role in the first entry sites (gills, skin, and intestine) for pathogens. Fish IL-17A and IL-17F show the same genomic organization as that of mammals, therefore named as IL-17A/F. Later on, IL-17N fish-specific ligands were described in the fish genome and known as a teleost-specific ligand [152]. The expression of genes from the IL-17 family was actively observed in the gills of common carp in response to *A. hydrophila* [86]. In the appointed observations, upregulation of most IL17 genes occurred at early stages of microbial challenging (4 h post infection) in gills and then declined slowly. The only genes that were substantially upregulated for a prolonged time (12 h post-infection) were IL17A/F2 and IL17N. In the most recent experiment, common carp interleukins (IL-17Na and b) were identified and confirmed through real-time polymerase chain reaction (real-time PCR) [153]. When carp were challenged with *A. hydrophila* infection, IL-17Ns were highly expressed in the brain tissue at 6 h post-infection; at day 1 post infection, the expression was upregulated in other tissues (head kidney, spleen, liver, and muscle) as well. In addition to that, the upregulation of the cytokines IL1β, IFN-γ, and IL-6 and chemokine CCL were also noticed post infection. In conclusion, the varying pattern of expression for different genes in the IL-17 family during *A. hydrophila* infection in gills and other tissue of carp indicates a pivotal role performed by these genes in regulating early immune responses.

Upon IFNs discovery, their biological function was first attributed to antiviral mechanisms, but later on, IFNs acquisition of antibacterial activity was also recognized [154]. Interferons inhibit bacterial infection by recognizing Toll-like receptors (TLR) and/or pattern recognition receptors (PRRs) present on infected cells [19]. These receptors have been found to bind molecules from a range of bacterial cellular components such as lipopolysaccharides, polysaccharides, and bacterial DNA to initiate the production of IFN genes controlling the infection [155]. Following these discoveries, a revolution of genetic identification and characterization for various IFNs regulatory genes aided in promoting the study of their gene expression during diseases' development.

Interferon regulatory factors (IRFs) play an important role in regulating both type 1 IFN and IFN-stimulated genes [89]. They are crucial in the innate immune response that regulates the expression of IFNs and initiate an antiviral and antibacterial response in teleost. For instance, carp challenged with *A. hydrophila* and polyinosinic:polycytidylic acid (poly I:C) stimulation upregulated IRF9 expression in the spleen, head kidney, foregut, and hindgut at different time intervals. Furthermore, transfection of IRF9 with poly I:C and LPS stimulation upregulated the expression of cytokines, including interferon-stimulatory genes (ISG)15, type 1 IFN, and TNFα in epitelioma papulosum cyprini (EPC) cells [88]. This finding suggests the potential antiviral and antibacterial role of IRF9 in fish. Furthermore, the so-called carp interferon regulatory gene (CcIRF10) apparently participates in stabilizing the bacterial infection via negative regulation of IFN response [89]. This inhibitory effect of IRF10 on IFN has been similarly described in a study on zebrafish infected with SVCV [156]. The authors of the underlying study linked their findings to the possible strategy of the

immune system to avoid excessive immunopathological reactions resulting in response to the viral infection. However, documenting the same effect in the absence of viral intrusion, as in the study conducted by Zhu 2020 et al. [89], may further explain an intrinsic role of IRF10 in carp following the onset of bacterial infection. The implicated mechanism of interferon inhibition by IRF10 might have taken place to suppress the development of severe inflammation or as a proactive tactic to reduce the implying of potential secondary viral infection. In this regard, examining the specific role of IRF10 in carp co-infected with *A. hydrophila* and SVCV might be an issue of interest for future research orientation. Upon review, these findings suggest that interferon regulatory factors play a significant role in regulating type 1 IFN and ISG genes in common carp to *A. hydrophila* infection. Further understanding the levels and activity of IRF genes in common could be the future interest.

4. Conclusions

Cytokines are essential components in the innate immune system, which play a crucial role in maintaining fish health. Their immunoreactive function in common carp to studied pathogens gives us an insight into what may have been the minimal or even the absolute cytokine network needed to initiate innate and adaptive immune responses. The information presented in this review article indicates that cytokines, including interferons, interleukins, and tumor necrosis factors, play an important role in the clearance of pathogens and the enhancement of antimicrobial activities in carp. Hence, analyzing and assessing their expression profile in common carp to the diseases mentioned above could be an advantage for developing vaccines for aquaculture. The type of cytokine in common carp and its induced response can depend on the nature of infectious agents. Variation of cytokine responses depends on the disease, as demonstrated by the differences between interferons and interleukins produced when infected with cyprinid herpesvirus 3, rhabdovirus, and *A. hydrophila* infections, respectively. However, the inconsistent expression of cytokines could also be due to the different genetic backgrounds of carp variants. Therefore, the genetic diversity of carp strains and their cytokine response to pathogenic stimuli is a domain where much remains to be discovered. In conclusion, a deeper understanding of the cytokine profile in common carp associated with various infectious agents is a continuing research demand.

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CHAPTER 3

IMMUNE RESPONSES IN CARP STRAINS WITH DIFFERENT SUSCEPTIBILITY TO CARP EDEMA VIRUS DISEASE

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Immune responses in carp strains with different susceptibility to carp edema virus disease

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ABSTRACT

Carp edema virus disease (CEVD), also known as koi sleepy disease (KSD), represents a serious threat to the carp industry. The expression of immune-related genes to CEV infections could lead to the selection of crucial biomarkers of the development of the disease. The expression of a total of eleven immune-related genes encoding cytokines (IL-1β, IL-10, IL-6a, and TNF-α2), antiviral response (Mx2), cellular receptors (CD4, CD8b1, and *GzmA*), immunoglobulin (IgM), and genes encoding-mucins was monitored in gills of four differently KSD-susceptible strains of carp (Amur wild carp, Amur Sasan, AS; Ropsha scaly carp, Rop; Prerov scaly carp, PS; and koi) on days 6 and 11 post-infection. Carp strains were infected through two cohabitation infection trials with CEV genogroups I or IIa. The results showed that during the infection with both CEV genogroups, KSD-susceptible koi induced an innate immune response with significant up-regulation ($p < 0.05$) of IL-1β, IL-10, IL-6a, and TNF-α2 genes on both 6 and 11 days post-infection (dpi) compared to the fish sampled on day 0. Compared to koi, AS and Rop strains showed up-regulation of IL-6a and TNF-α2 but no other cytokine genes. During the infection with CEV genogroup IIa, Mx2 was significantly up-regulated in all strains and peaked on 6 dpi in AS, PS, and Rop. In koi, it remained high until 11 dpi. With genogroup I infection, Mx2 was up-expressed in koi on 6 dpi and in PS on both 6 and 11 dpi. No significant differences were noticed in selected mucin genes expression measured in gills of any carp strains exposed to both CEV genogroups. During both CEV genogroups infections, the expression levels of most of the genes for T cell response, including CD4, CD8b1, and *GzmA* were down-regulated in AS and koi at all time points compared to day 0 control. The expression data for the above experimental trials suggest that both CEV genogroups infections in common carp strains lead to activation of the same expression pattern regardless of the fish's susceptibility towards the virus. The expression of the same genes in AS and koi responding to CEV genogroup IIa infection in mucosal tissues such as gill, gut, and skin showed the significant up-regulation of all the cytokine genes in gill and gut tissues from koi carp at 5 dpi. Significant down-regulation of CD4 and *GzmA* levels were only detected in koi gill on 5 dpi but not in other tissues. AS carp displayed significant up-expression of Mx2 gene in all mucosal tissues on 5 dpi, whereas in koi, it was up-regulated in gill and gut only. In both carp strains, gill harbored a higher virus load on 5 dpi compared to the other tissues. The results showed that resistance to CEV could not

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be linked with the selected immune responses measured. The up-regulation of mRNA expression of most of the selected immune-related genes in koi gill and gut suggests that CEV induces a more systemic mucosal immune response not restricted to the target tissue of gills.

Subjects Aquaculture, Fisheries and Fish Science, Veterinary Medicine, Virology, Zoology, Freshwater Biology
Keywords Carp edema virus, Common carp, Gene expression, Immune related genes, Mucosal response

INTRODUCTION

Common carp rearing condition has intensified in recent decades, which could have resulted in increased chronic stress and a weakened immune system, increasing their susceptibility to various pathogens. Among them, infection caused by carp edema virus (CEV) is gaining attention, as it is characterized by high mortality rates but can be also manifested as subclinical infection (*Amita et al., 2002*; *Ono, Nagai & Sugai, 1986*). Therefore the disease caused by CEV and known as carp edema virus disease (CEVD), or koi sleepy disease (KSD) could represent a significant threat to the carp industry. The KSD name has been derived from the main clinical signs manifested by affected fish which usually show lethargy and increasing unresponsiveness, as they can be seen lying in the bottom of tanks for extended periods (*Pretto et al., 2013*). Gross lesions incorporating spreading hemorrhagic skin lesions with edema, particularly in the abdomen, pale gills, sunken eyes, and ulcerative inflammation on the anus may also be seen (*Oyamatsu et al., 1997*). A large amount of mucus is produced on the skin and gills as well (*Zhang et al., 2017*). The CEVD/KSD was first reported in koi farms in Japan in 1974, where it caused substantial mortalities (*Amita et al., 2002*; *Ono, Nagai & Sugai, 1986*) and economic losses. Since then, the disease has spread almost worldwide (*Machat et al., 2021*).

Carp edema virus (CEV), which belongs to the Poxviridae family, has a mulberry-like structure made up of double-stranded DNA about 250–280 nm in diameter (*Oyamatsu et al., 1997*). The infection seems to be most prevalent in the gills. An electron microscope revealed that diseased fish exhibit morphologically altered gill epithelium that displays poxvirus-like structures (*Jung-Schroers et al., 2015*; *Miyazaki, Isshiki & Katsuyuki, 2005*; *Haenen et al., 2014*; *Pretto et al., 2013*). Two to three different genogroups (genetic clades) of CEV have been so far characterized: I, IIa, and IIb (*Way & Stone, 2013*; *Matras et al., 2017*; *Adamek et al., 2017b*). Common carp are the primary hosts of genogroup I, which has been detected in most European waters. A majority of genogroup IIa reports have been in koi, but not exclusively, while genogroup IIb has been detected in both carp and koi samples (*Matras et al., 2017*; *Adamek et al., 2018*; *Matějíčková et al., 2020*; *Ouyang et al., 2020*).

Like higher vertebrates, teleosts have an immune system that employs both specific (adaptive) and non-specific (innate) responses against pathogens such as viruses, bacteria, and parasites (*Whyte, 2007*). The non-specific immune response is considered the very first

defence against pathogens. Unlike other vertebrates, fish primarily rely on the non-specific immune system for survival during early embryonic development (*Rombout et al., 2005*). The fact that fish are poikilothermic also means they rely less on some of the conventional characteristics of adaptive immunity, due to slow and relatively low antibody defences and slow proliferation, maturation, and memory of lymphocytes. Thus, it is considered that innate immune responses play a fundamental role in fish immunity.

In higher animals infections with poxviruses are contained through combination of innate and adaptive immunity. The response to infection is primary undertaken by the inflammatory and natural killer (NK) cells (*Smith & Kotwal, 2002*). These non-specific responses control viral replication and allow the time for mounting a specific antigenic adaptive response (*Magnadottir, 2010*). For instance, T lymphocytes, also referred to as T cells, are a crucial component of the adaptive immune response to infections. They are a type of white blood cell that have the ability to recognize and respond to viral antigens and regulate the immune response through cytokine production (*Andersen et al., 2006*; *Tortorella et al., 2000*). Research has indicated that T cell responses play a vital role in controlling pox virus infections, and the activation and expansion of T cells is a significant factor in resolving the infection (*Yamaguchi et al., 2019*). Furthermore, genetic variations in fish have been shown to affect the T cell response magnitude and specificity to pox viruses, which may impact the outcome of the infection (*Adamek et al., 2021*). During CEV infection in carp the immune response is somehow complicated by onset of environmental immunosuppression caused by intoxication with ammonia (*Adamek et al., 2021*), however the host–virus interaction is reciprocally impacted by host genetic competitiveness and virus genomic characteristics. Several emerging genogroups of CEV have so far been identified among carp populations worldwide. These genogroups are seemingly selective mutations targeted toward the numerous variants within the common carp strains/species (*Adamek et al., 2017c*). It has been also reported, that there are differences between various strains of carp in the expression of cytokines during viral infections, specifically the interferon (IFN) and interferon-stimulated genes (ISGs) (*Tadmor-Levi et al., 2019*). Several varieties of common carp have emerged as an outcome of natural geographic separation of common carp groups and domestication, providing a wide range of genetic resources. As already has been published, there may be a large variations in susceptibility among strains that have a different genetic background, as was evident in studies focused on evaluation of susceptibility of different common carp strains to experimental infection of cyprinid herpesvirus 3 (CyHV-3) (*Shapira et al., 2005*; *Rakus et al., 2009*; *Piačková et al., 2013*; *Adamek et al., 2019*). Comparatively to other viral diseases in fish, scant studies have been conducted on the gene expression patterns of immune-related genes in CEV-infected carp strains. In case of CEV, researchers have exposed different strains of carp (Amur wild carp, Amur sasan, AS; Ropsha scaly carp, Rop; Prerov scaly carp, PS; and koi), having different susceptibility to CEV, genogroups I and IIa (*Adamek et al., 2017a*). However, the study focused primarily on determining whether carp strains were susceptible to the disease, and only type I interferon responses as the parameter of non-specific immunity were assessed for CEV affected carp strains. The mucosal-epithelial barrier is a vital component of the innate immune response of fish against viruses (*Langevin et al., 2013*; *Secombes & Zou, 2017*). It

includes the gills, skin, and gastrointestinal tract as its main components (*Huttenhuis et al., 2006*; *Magnadottir, 2010*), all of which encounter pathogenic agents present in the aquatic environment. This barrier employs various mechanisms to hinder pathogen invasion, including the release of antimicrobial factors by immune cells or tissues. The gut mucosal layer serves as the first barrier against pathogen invasion, but it also provides nutrients for bacterial pathogens (*Garcia et al., 1997*), resulting in a delicate balance between gut mucosal secretions and microorganisms. Studies on innate immune responses of fish gut epithelial cells against viral infections are limited. Carp gut epithelial cells exhibit increased expression of cytokines such as IFN-α2, IL-1 β, and iNOS upon cyprinid herpes virus 3 infection, highlighting the significance of gut epithelial cytokine signalling in maintaining mucosal immunity (*Syakuri et al., 2013*). The skin serves as the primary line of defence against invading pathogens and plays a critical role in immune responses. However, the molecular mechanisms underlying the fish skin's immune response remain poorly understood, and its potential as an indicator of immune competence is unknown, despite the convenience of non-invasive skin sampling. The expression of immune-related molecules in fish skin could significantly contribute to the immune response against infections. Some observed differences in cytokine genes in disease susceptibility between fish species and/or strains have been linked to the differing ability of the fish to prevent pathogen attachment and entry at mucosal epithelial sites (*Adamek et al., 2019*; *Adamek et al., 2022a*; *Adamek et al., 2022b*). The gill mucosal immune system is distinguished by various humoral and cellular immune mechanisms that synergize to safeguard the tissue against infection (*Gomez, Sunyer & Salinas, 2013*). When infected, both the local immune cell populations, which include mucosal "innate" T-cells and IgT + B cells, and the immune cells mobilized from specialized immune organs *via* the bloodstream, can contribute to the elimination of the infection (*Marcos-López et al., 2017*).

The primary constituents of the mucus layer are large, filamentous glycoproteins known as mucins that are highly glycosylated. They are strongly adhesive and play a crucial role in protecting the mucosal surfaces (*Roussel & Delmotte, 2005*). Mucins impart viscosity to mucus and form a framework within which various antimicrobial molecules are present (*McGuckin et al., 2011*). As mentioned earlier, excessive mucus production during CEV infection is one of the major clinical symptoms. Therefore, conducting experimental trials to understand the role of mucin in fish affected by CEV would be interesting.

It has been proven that gills are the known target tissue of CEV and are crucial for the biology of the all viral genogroups (*Adamek et al., 2017c*). Therefore, in the present study, the initial part consists of an evaluation of immune gene responses to CEV genogroup I and IIa infections in the gills of different carp strains (AS, koi, PS, and Rop). In addition, to determine whether or not mucosal immune genes fully respond to CEV infection, therefore, two carp strains (KSD-susceptible koi and KSD-resistant AS) were exposed to CEV genogroup IIa. In the experimental trial previously published by *Adamek et al. (2017c)*, the expression of type I interferon and interferon-stimulated genes encoding IFNa2 were reported in the gills of common carp strains. The background and a brief summary of CEV load and replication for the study groups were compiled from previous experimental trials and can be found in Tables S1 & S2. Here, we focus on the Co II and

Co IV infectious trials, which were infected with genogroup I and IIa, respectively, and from which the samples were analyzed. In the current study, to measure changes in gene expression of selected immune genes, reverse transcription-quantitative PCR (RT-qPCR) assays have been used. Our data provide a deeper understanding of CEV pathogenesis through cytokine and other immune-related genes.

MATERIALS & METHODS

Infections with CEV genogroup I and IIa

Experimental samples acquired

The samples analyzed in the present study are comprised of two experimental parts. According to the 3R rule, the samples obtained during experimental infections were reused for additional analysis of the immune responses. The experimental procedures were approved by Lower Saxony State Office for Consumer Protection and Food Safety (LAVES), Oldenburg, Germany under the reference number: 33.19–425 2-04-16/2144. In the initial part, the samples used for analysis originated from the previously published infection trial of CEV in different carp strains (*Adamek et al., 2017c*). Naïve recipient common carp strains Amur wild carp (AS), Ropsha scaly carp (Rop), Prerov scaly carp (PS), and koi were acquired as swimming fry from the University of South Bohemia in Ceske Budejovice, Faculty of Fisheries and Protection of Waters, located in Vodnany, Czech Republic. Using a full-factorial mating scheme of three females with three males, each experimental stock was established by artificial reproduction of the appropriate carp strain (*Kocour et al., 2005*). From the time the eggs were laid, the stocks were maintained in a closed recirculation system that was supplied with tap water. Later on, fry were transported to the facility of the University of Veterinary Medicine in Hannover and raised in a recirculation system at 20 °C in tap water. Commercial carp feed (Skretting, Norway) was fed to fish at 1% of body weight per day. The average weight of the fish at the beginning of the infection experiments was 3.7 ± 0.9 g were kept under virus and parasite-free conditions. Each carp population was confirmed to be free of DNA/RNA specific for CyHV-3, spring viremia of carp virus (SVCV), and CEV by the mean of RT-qPCR or qPCR before using them in infection experiments.

The infections of tested fish have been carried out by cohabitation with either CEV-affected common carp or koi showing clinical signs of the disease. Both CEV-affected common carp and koi were examined by the mean of end-point PCR (already published data) (*Adamek et al., 2017c*) which confirmed CEV genogroups I and IIa, respectively.

Sample analysing theme for the experimental part one

Total four cohabitation experiments (Co I, II, III, and IV) were performed with all four strains. More detailed information for all the infectious trials mentioned above can be found in *Adamek et al. (2017c)*. The present study was only focused on the samples from cohabitation experiment II (Co II) and IV (Co IV). In the Co II trial, 11 individuals from all four strains AS, koi, PS, and Rop were cohabited with CEV genogroup I affected common carp. On days 6 and 11 post-infection, four fish from each strain were euthanized by immersion into a 0.5 g L-1 tricaine (Sigma) solution. The samples from the gills were

collected individually in RNAlater for RNA isolation and gene expression analysis. During the Co IV experiment, the same four carp strains (eight fish per strain) were cohabited with koi infected with CEV genogroup IIa. Subsequently, four fish from each strain were euthanized with the above-mentioned method at days 6 and 11 post-infection. Their gills were collected in RNAlater. Non-infected fish from each strain were used as a negative control for gene expression in both experiments.

Fish rearing and sample analysing theme for the second experiment part

In the second experimental study, naïve AS and koi with an average body weight of 20.4 ± 10.9 g were used to evaluate the selected immune-related gene expressions in the mucosal organs to CEV infection. Before the start of the experimental infection trial, fish were kept in the similar condition mentioned above and confirmed to be free from DNA/RNA specific for CyHV-3, spring viremia of carp virus (SVCV) and CEV. Feeding was done using a commercial feed (Perla Plus, Skretting, Norway) at a rate of 1% body weight per day. In this part of the study, the data were collected as described by *Adamek et al. (2017c)*. Total of five fish from each strain were cohabitated with koi infected with CEV genogroup IIa. At day 5 post-infection, fish from both strains were euthanized by immersion into a 0.5 g L^{-1} tricain (Sigma) solution, and mucosal tissues such as gill, gut and skin were collected in RNAlater. Fish of the same strains sampled in the day 0 were used as a non-infected control for gene expression analysis.

DNA isolation

DNA was isolated as previously described in *Adamek et al. (2017c)*. Specifically: 25 mg of tissue was mechanically lysed in a QIAgen Tissuelyser II (Qiagen, Hilden, Germany), then the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions was used. After isolation, the samples were diluted to 50 ng μL −1 and stored at −80 °C.

RNA extraction and cDNA synthesis

Total RNA was extracted from the 25 mg of RNAlater-stored tissue samples using Tri-reagent (Sigma, St. Louis, MO, USA), according to the manufacturer's instructions. The remaining genomic DNA was digested with 2 U of DNase I according to the manufacturer's protocol. Prior to cDNA synthesis, RNA concentrations were determined by spectrophotometry, the integrity was checked using a 1.5% agarose gel. Complementary DNA (cDNA) was synthesized from 300 ng of total extracted RNA using Maxima TM First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). Prior to RT-qPCR analysis, cDNA samples were diluted 1:20 with nuclease-free water. Concentrations of the samples were determined so that homogeneous RNA could be produced for cDNA synthesis. cDNA samples were stored in −20 °C until further use.

RT-qPCR/qPCR analysis

For the estimation of CEV load from CEV genogroup IIa, a qPCR-based double-labelled probe was used as described by *Adamek et al. (2017b)*. RT-qPCR assays were carried out using synthesized cDNA and specific primers listed in Table 1 to examine the expression of

all selected immune-associated genes in gills from part one of the experiment and gill, skin and gut from the second part of the experiment. The RT-qPCR reactions were carried out in duplicates using Maxima SYBR Green 2x master mix (Thermo Fisher Scientific, Waltham, MA, USA) in StepOne Plus Cycler (Thermo Fisher Scientific, Waltham, MA, USA). The reaction mixture was prepared as follows: 1 × Maxima SYBR Green mastermix (with 10 nM of ROX), 0.2 μM of each primer, 3.0 μL of 20 × diluted cDNA and nuclease-free water to a final volume of 10 μL. The following amplification program was used: initial denaturation (10 min at 95 °C) followed by 40 cycles of denaturation (30 s at 95 °C), annealing (30 s at 55 °C), and elongation (30 s at 72 °C). The non-template and minus reverse transcriptase (-RT) controls were performed for each reaction mix and cDNA sample, respectively. Obtained RT-qPCR data were analyzed using the StepOne software version 2.1 by measuring and analyzing the quantitative cycles (Cq) for every reaction and exported to Microsoft Excel. To determine the amount of particular gene copy numbers present in each sample, recombinant plasmid standard curve from 6×10^0 to 6×10^6 gene copies were prepared and used. To normalize expression, the 40S ribosomal protein S11 was used as a reference gene. Using the formula below, the level of gene expression was calculated as the copy number of the gene normalized against 1×10^5 copies of 40S ribosomal protein S11 (normalized copy number):

Normalized copy number = mRNA copies per PCR for target gene/(mRNA copies per PCR for reference gene/10^5).

Statistical analysis

Initially, raw RT-qPCR data were analyzed using StepOne software v2.1. The normalized gene expression data were transformed using a Log 10 (x) transformation before statistical analysis. Statistical analysis was performed using SigmaPlot 12.5 software (Systat Software, Chicago, IL, USA). To detect significant differences ($p \leq 0.05$) in gene expression and viral load during CEV infection, 1-way or 2-way ANOVA were used along with pairwise multiple comparisons using the Holm-Sidak method.

RESULTS

CEV viral load and replication

The viral load and replication of the samples have been determined and published previously (*Adamek et al., 2017c*). The common carp strains infected with CEV affected carp with genogroup I manifested significant differences in susceptibility to the infection. Koi and PS were more susceptible than AS and showed a high viral load and CEV mRNA expression than other strains. For additional details, please refer to Table S1.

When the common carp strains and naïve koi were infected with CEV genogroup IIa (experiment Co IV), koi showed the highest viral load from all strains in both sampling times (151,668 mean copies at 6 dpi, 1,253,267 copies at 11 dpi), which confirmed its higher susceptibility to this genogroup. See Table S2 for more information.

Table 1 RT-qPCR primers used in the study.

Target gene	Sequences	GeneBank ID or reference
IL-10	CGCCAGCATAAAGAACTCGT	AB110780
	TGCCAAATACTGCTCGATGT	
IL-1 β	AAGGAGGCCAGTGGCTCTGT	
	CCTGAAGAAGAGGAGGCTGTCA	AJ245635
IL-6a	CAGATAGCGGACGGAGGGGC	
	GCGGGTCTCTTCGTGTCTT	KC858890
TNF- α2	CGGCACGAGGAGAAACCGAGC	
	CATCGTTGTGTCTGTTAGTAAGTTC	AJ311801
Mx2	ATGACCCAGCAGAAGTGGAG	
	CAGGAACATTGGCAGAGATG	XM_019081222
IgM	CACAAGGCGGGAAATGAAGA	
	GGAGGCACTATATCAACAGCA	AB004105
CD4	CGTGGACATCTGGCTTTGTG	
	TTTGGTTTTGCGTCGTCTGT	DQ400124
CD8b1	CGGCTCGGAAACTATCACCT	
	GAGTGGCGGACAGGTTTTCTC	EU025120
GzmA	GTGTTGGCATCGTCAGTTACG	
	AGTACCCCAACCTGTCACG	GU362096
40S	CCGTGGGTGACATCGTTACA	AB012087
	TCAGGACATTGAACCTCACTGTCT	
Muc5b	CAGCCCTCTTCCTCTTTCATC	
	CCACTCATCTTTCCTTTCTCTTC	*Van der Marel et al. (2012)*
Muc2c	TGACTGCCAAAGCCTCATTC	
	CCATTGACTACGACCTGTTTCTC	

Expression of IL-10, IL-1 β, TNF-α2 and IL-6a genes in carp strains during the infection with CEV genogroups I and/or IIa

In the challenge with CEV genogroup I (Co II), koi depicted significant lower level of IL-10 and higher levels of IL-6a on day 6th post-infection compared to day 0 p.i. On 11th dpi, koi showed significantly higher level of IL-1β, TNF-α2 and IL-6a genes, and significant down-regulation of IL-10 gene expression compared to day 0 (Fig. 1). Whereas, in AS, the expression of IL-10 was at the same level on both days 6 and 11 p.i. compared to day 0. Significant up-regulation of IL-1β, and IL-6a genes were noticed in AS only on day 11 p.i. Significant elevated levels TNF-α2 were found on both days 6 and 11 p.i. In PS strain, expression of IL-1β was significantly up-regulated on day 6 p.i. However, no significant differences were detected in the expression of other immune genes such as IL-1β, TNF-α2 and IL-6a on both time points compared to fish sampled on day 0. In Rop, significant up-regulation of TNF-α2 and IL-6a was observed on both days 6 and 11 p.i. However, there was no differences in the expression of IL-10, and IL-1β on both sampling days.

During the course of an infection with CEV genogroup IIa (Co IV), koi evinced significant up-regulation of IL-10 and IL-1β on both days 6 and 11 p.i. compared to day

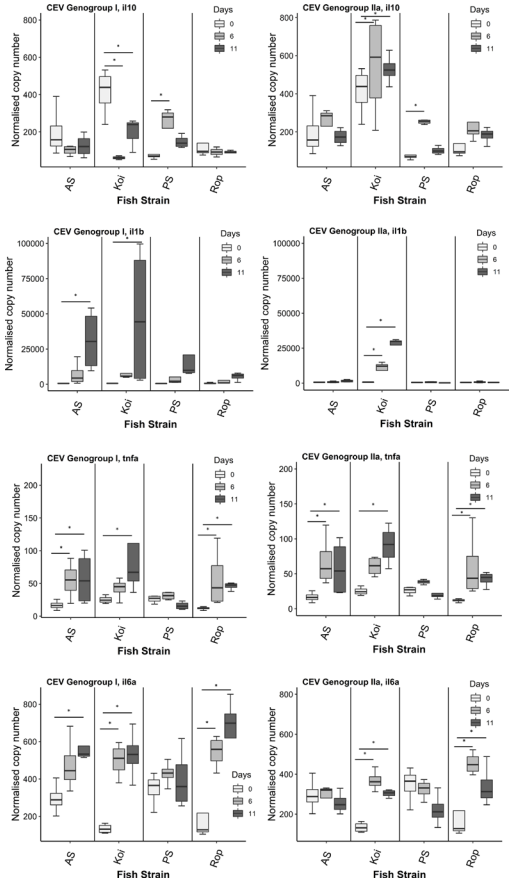

Figure 1 Reverse transcriptase quantitative PCR (RT-qPCR) analysis of IL-10, IL-1 β, TNF- α2 and IL-6a genes in gills of four strains. Gene expression was measured in the gill of carp individuals from different strains (AS, koi, PS, and Rop) at days 0 (non-infected), 6 and 11 post-infections of CEV genogroup I or IIa. The gene expression was normalized to the expression of the gene encoding the S11 protein of the 40S subunit as a reference gene. Data are shown as box plot indicating mean and standard deviation from $n = 4$ fish. Asterisks denote statistically significant differences ($^*p < 0.05$) between the control (day 0) and infected once (day 6 and 11).

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0. On day 6 p.i., TNF-α2 levels was not significantly higher on 6 dpi and the increase continued to 11 dpi, when it was significantly higher than control (0 dpi). The expression level of IL-6a was significantly higher on day 6 p.i., then it gradually decreased but remained significantly up-regulated also on day 11 p.i. comparing with day 0. In AS, the expression of IL-10 gene was at the same level on both days 6 and 11 p.i. compared to day 0. No significant differences were observed also in IL-1β levels on both days 6 and 11 p.i. Significantly higher level of TNF-α2 was noticed on both days 6 and 11 p.i., and these levels were much higher when compared to genogroup I infection in AS. In PS strain, only expression of IL-1β was significantly up-regulated on day 6 p.i. compared to day 0. No significant differences were detected in the expression of other immune genes such as IL-1β, TNF-α2 and IL-6a on both time points compared to fish sampled on day 0. In Rop, significant up-regulation of TNF-α2 gene on both days. The expression of IL-6a gene was also up-regulated on days 6 and 11 p.i. with slight decrease between 6 and 11 dpi. Furthermore, there was no differences in the expression of IL-10, and IL-1β on both days 6 and 11 p.i.

Expression of CD4, CD8b1 and GzmA in carp strains during the infection with CEV genogroups I and/or IIa

Challenge with CEV genogroup I (Co II), significantly down-regulated the expression of CD4 and *GzmA* in koi on days 6 and 11 p.i. compared to day 0 (Fig. 2). We did not observe any differences in the expression of CD8b1 in koi on both days 6 and 11 p.i. AS showed remarkable significant down-expression of CD8b1 and *GzmA* genes on both infected days 6 and 11 compared to day 0. In addition, the expression level of CD4 was also significantly down-regulated on days 6 and 11 p.i. but the response was not stronger as CD8b1 and *GzmA* genes when compared to day 0. Furthermore, PS and Rop strains did not show any significant differences in the expression of CD4, CD8b1 and *GzmA* on day 6 and 11 p.i. compared to fish sampled on day 0.

In the challenge with CEV genogroup IIa (Co IV), koi manifested significant down-regulation of CD4 on day 6 and 11 p.i. compared to day 0. However, there was no CD8b1 response detected in koi on both infected days compared to control group. The gradual decrease of *GzmA* was noted in koi on both day 6 and 11 p.i compared to day 0, but with significant difference on day 11 only. There was no difference in CD4 expression in AS strain p.i compared to day 0. However, significant down-regulation of CD8b1 gene expression was noticed in AS on both days 6 and 11 p.i when compared to day 0. Furthermore, slight down-regulation of *GzmA* was seen but without significant difference. In PS and Rop no significant differences were observed in the expression of CD4, CD8b1 and *GzmA* on both time points compared to fish sampled on day 0.

Expression of Mx2, IgM, and Mucin genes in carp strains during the infection with CEV genogroups I and/or IIa

During the challenge with CEV genogroup I (Co II), the significantly higher expression level of Mx2 was noticed in koi on day 6 p.i. however on day 11 p.i. the levels were restored near day 0 (Fig. 3). The expression of IgM in koi was significantly down-regulated on day 11 p.i. compared to day 0. On day 6 p.i., IgM levels was also lower but not significant when compared to control group. In AS, on both days 6 and 11 p.i. we did not observe

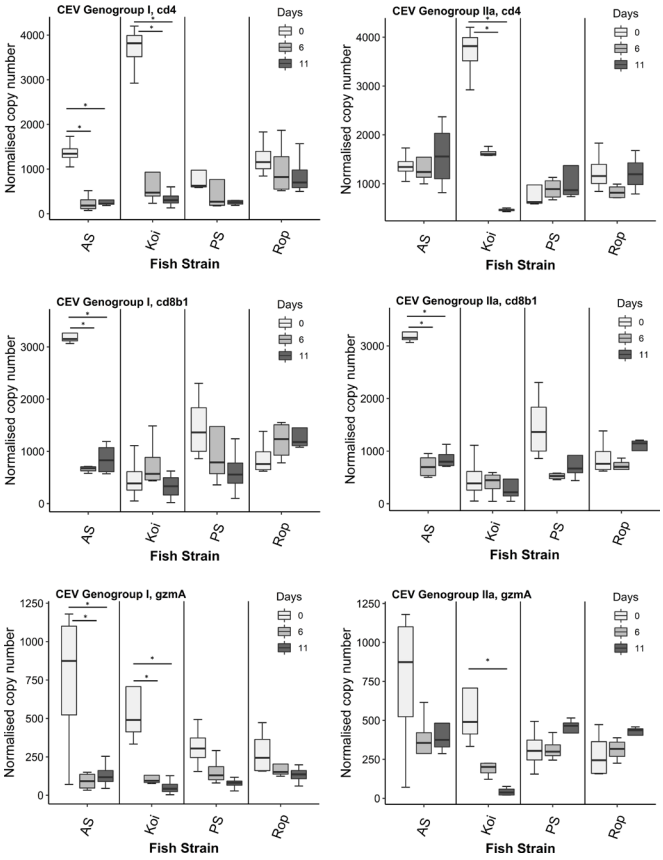

Figure 2 Reverse transcriptase quantitative PCR (RT-qPCR) analysis of CD4, CD8b1 and *GzmA* genes in gills of four strains. Gene expression was measured in the gill of carp individuals from different strains (AS, koi, PS, and Rop) at days 0 (non-infected), 6 and 11 post-infections to CEV genogroup I and IIa. The gene expression was normalized to the expression of the gene encoding the S11 protein of the 40S subunit as a reference gene. Data are shown as box plot indicating mean and standard deviation from $n = 4$ fish. Asterisks denote statistically significant differences ($^*p < 0.05$) between the control (day 0) and infected once (day 6 and 11).

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any significant difference in the expression of Mx2 gene compared to day 0. The IgM levels in AS was significantly down-expressed on day 6 p.i. compared to control fish. Mx2 expression in PS strain was significantly peaked on day 6 p.i. and stayed elevated up to day

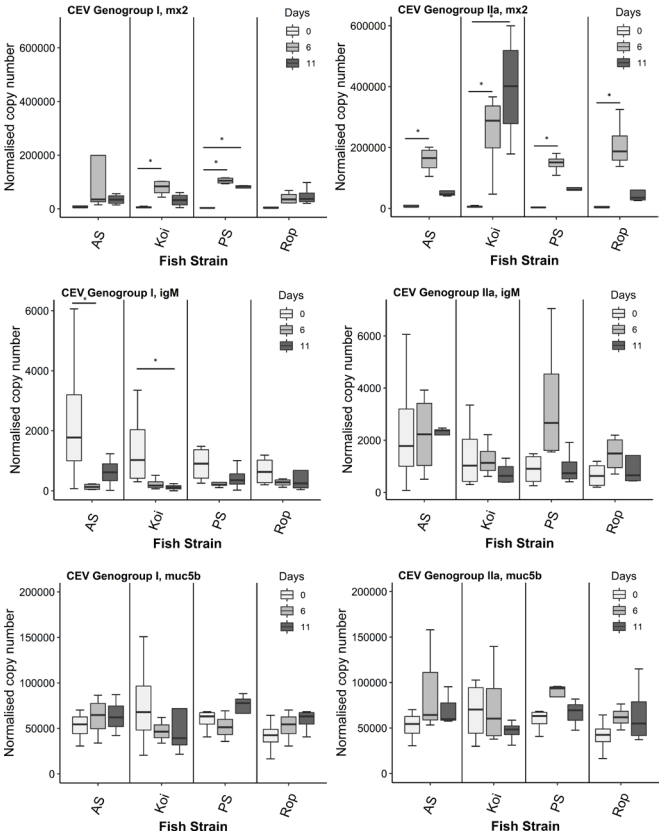

Figure 3 Reverse transcriptase quantitative PCR (RT-qPCR) analysis of Mx2, IgM, and Mucb5 in gills of four strains. Gene expression was measured in the gill of infected and non-infected (day 0) carp individuals from different strains (AS, koi, PS, and Rop) at days 6 and 11 post-infection to CEV genogroup I and IIa. The gene expression was normalized to the expression of the gene encoding the S11 protein of the 40S subunit as a reference gene. Data are shown as box plot indicating mean and standard deviation from $n = 4$ fish. Asterisks denote statistically significant differences ($*p < 0.05$) between the control (day 0) and infected once (day 6 and 11).

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11 p.i. compared to control group. No IgM response was detected in PS strain on both infected days. Rop did not exhibit a significant shift in expression level of Mx2 or IgM on either day 6 or day 11 p.i compared it to control.

Table 2 **CEV virus load in tissues from AS and Koi after experimental cohabitation with CEV genogroup IIa.**

	AS Day 5	Koi Day 5
Gill		
Mean	2.09E + 04[a]	2.51E + 05[bc]
Median	7.86E + 03	2.52E + 05
SD	3.09E + 04	9.68E + 04
Gut		
Mean	1.08E + 02[c]	1.83E + 03[c]
Median	3.20E + 01	1.34E + 03
SD	1.96E + 02	2.05E + 03
Skin		
Mean	5.29E + 02[c]	1.10E + 05[b]
Median	1.21E + 02	7.50E + 04
SD	1.08E + 03	1.10E + 05

Notes.

Carp edema virus load was measured by qPCR as copy numbers of virus specific DNA in the gill, gut and skin of AS and koi during CEV genogroup IIa infection. Samples were collected 5-day post-exposure from $n = 6$ fish per day. The data on virus load is shown as mean, median and standard deviation (SD) of genome copies in 250 ng of isolated DNA. Different letters indicate significant differences at $p \leq 0.05$ between carp strains.

Upon challenged with CEV genogroup IIa (Co IV), in koi there was observed a significant upward trend of Mx2 gene on both days 6 and 11 p.i. in comparison to the control day 0. The IgM expression in koi gradually slightly decreased but without statistical significance. In AS, the significant peak level of Mx2 was noticed on day 6 p.i. and reduced on day 11 p.i. near to the control group. There were no differences found in IgM transcripts in AS at both infected days compared to control fish. Both PS and Rop strains significantly up-regulated Mx2 expression levels on day 6 p.i. and then reduced to the level of control day 0. We did not observe any differences in IgM levels in both PS and Rop strains on both days 6 and 11 p.i. compared to the fish sampled on day 0. The present study also investigated the effects of CEV genogroup I or IIa exposure on Muc5b gene expression in the gills of four different fish strains. Results demonstrated that there was no significant difference in the Muc5b gene expression on both days 6 and 11 p.i. in the gills of all four strains when compared to the control. However, a non-significant slight decrease in the Muc5b mRNA level on day 11 was noted in the gills of koi carp following the exposure to CEV genogroup I or IIa, which differed slightly from the other fish strains.

Virus load in AS and koi carp infected with CEV genogroup IIa

The virus load was analyzed in the gill, gut and skin tissues at day 5 post-infection. Among all selected organs, gills harboured the highest number of CEV specific DNA copies with a mean of 251,130 copies and median of 252,444 in koi carp and mean of 20,934 and median 7,859 of copy numbers in AS per 250 ng of extracted DNA (Table 2). The skin of koi having the second highest viral load with a mean copy number of 110,457, and median 74,986, whereas the gut and skin organs from AS had a virus load below 1,000 copies per 250 ng of isolated DNA.

Expression of immune-related genes in the gill, gut and skin tissues of AS and koi during the infection with CEV genogroup IIa

IL-10, IL-1β, TNF-α2 and IL-6a genes

The expression level of all selected cytokine genes such as IL-10, IL-1β, TNF-α2 and IL-6a were significantly up-regulated in the gill and gut tissues of koi carp at day 5 p.i. compared to day 0 (Fig. 4). In contrast, the expression of none of the cytokine genes was shifted in both gill and gut tissue of AS. Only IL-10 gene showed slight lower expression in the gill on day 5 p.i. compared to day 0, but without a significant difference.

In the skin tissue, no significant changes were observed in cytokine genes expression in both koi and AS strains at day 5 p.i. compared to the fish sampled on day 0, except of IL-1β gene, which was significantly up-expressed ($p < 0.01$) at day 5 p.i. compared to day 0.

CD4, CD8b1 and GzmA genes

There was a significant down-regulation ($p < 0.05$) of CD4 and *GzmA* transcript levels observed in the gill of koi carp at day 5 p.i. compared to the control group (Fig. 5). However, no CD8b1 response was detected in koi at day 5 p.i., compared to the control day 0. In the AS strains' gill, slight down-regulation was observed in CD4, CD8b1, and *GzmA* levels on day 5 p.i. compared to day 0, although the levels were not statistically significant.

In the gut and skin of both carp strains, no significant changes were noticed in CD4, CD8b1, and *GzmA* transcripts on day 5 p.i. compared to the fish sample on day 0.

Mx2, IgM and Mucin genes

No significant difference in IgM levels were noticed in the gill and gut in both AS and koi strains on 5 dpi, compared to the control fish. Significant down-regulation of IgM was noticed in koi's skin at day 5 p.i. however, slight down-expression of IgM transcripts was found in the AS skin and gill tissue but without significant changes (Fig. 6).

The expression of the Mx2 gene in gill, gut and skin tissues in AS strain was significantly up-regulated on day 5 post-CEV exposure compared to day 0. The Mx2 transcripts in koi gill and gut were also significantly up-expressed on day 5 p.i. even much higher than AS strain. Further, koi skin tissue did not show any significant change on day 5 p.i. compared to the control.

The tissue-specific expression of mucin genes, Muc2 and Muc5b, was observed among gill, gut, and skin. Muc2 was exclusively expressed in the gut tissue, whereas Muc5b was expressed in the gill and skin tissues. No significant differences were found in the expression levels of these genes in gills and gut. However, a significant down-regulation of Muc5b transcripts, was observed in the skin tissues of koi on day 5 post-infection compared to the control.

DISCUSSION

Immune gene expressions during the infections with CEV genogroup I and IIa

The differences in the immune responses occurring between the fish strains with different susceptibility to pathogen could explain how the fish successfully protect themselves from

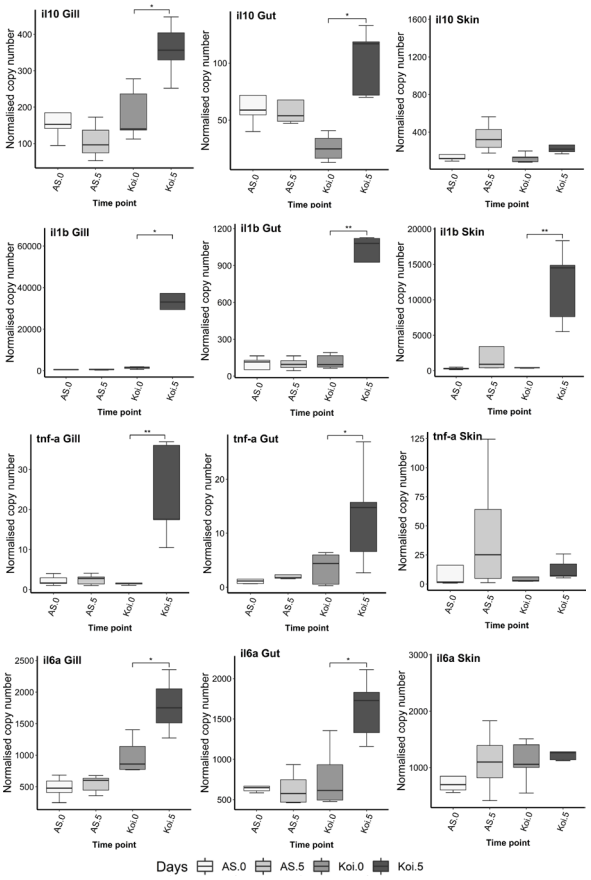

Figure 4 Reverse transcriptase quantitative PCR (RT-qPCR) analysis of IL-10, IL-1 β, TNF- α2 and IL-6a genes in the gill, gut and skin tissues of AS and koi at day 0 (non-infected) and 5 post-infection of CEV genogroup IIa. The gene expression was normalized to the expression of the gene encoding the S11 protein of the 40S subunit as a reference gene. Asterisks denote statistically significant differences marked with * at $p < 0.05$, with ** $p < 0.01$ between the control and infected once.

Full-size DOI: 10.7717/peerj.15614/fig-4

the infections. However, for fish poxviruses there is underwhelming scarcity of data. The immune responses were addressed only in handful of works; with only type I interferon response characterized during CEV infection in more than two carp strains (*Adamek et al.*,

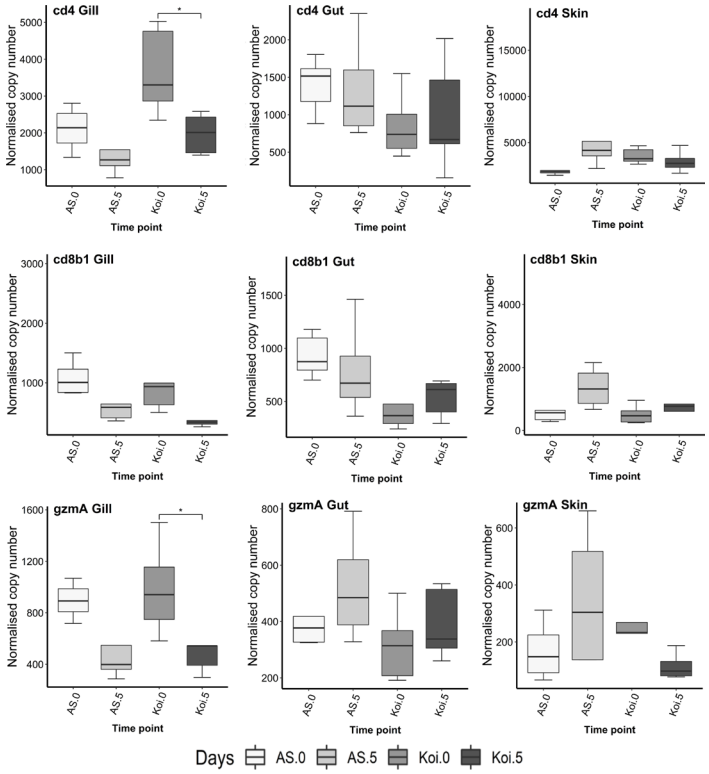

Figure 5 Reverse transcriptase quantitative PCR (RT-qPCR) analysis of CD4, CD8b1 and *GzmA* in the gill, gut and skin tissues of AS and koi at day 0 (non-infected) and 5 post-infection of CEV genogroup IIa. The gene expression was normalized to the expression of the gene encoding the S11 protein of the 40S subunit as a reference gene. Asterisks denote statistically significant differences marked with * at $p < 0.05$, with ** $p < 0.01$ between the control and infected once.

Full-size DOI: 10.7717/peerj.15614/fig-5

2017c). Furthermore, no studies describing the any other immune responses of carp strains after infection by cohabitation with fish infected with the CEV genogroup I or IIa have been reported yet. Therefore, our study is the first to report the looking on inflammation and adaptive immune responses in carp strains during CEV infections.

Cytokines belong to the most widely studied group of molecules involved in the function of the immune system. Amongst, a key pro-inflammatory cytokine, interleukin-1β (IL-1β) plays an essential role in the fish immune system (*Zou & Secombes, 2016*). According to the

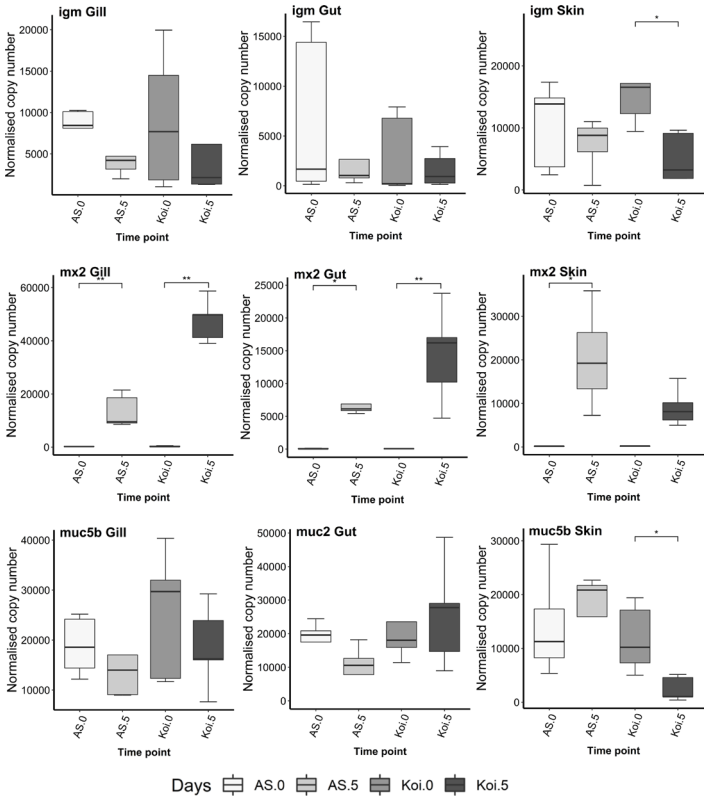

Figure 6 Reverse transcriptase quantitative PCR (RT-qPCR) analysis of Mx2, IgM, Muc2, and Mucb5 in the gill, gut and skin tissues of AS and koi. RT-qPCR analysis of Mx2, IgM, Muc2, and Mucb5 in the gill, gut and skin tissues of AS and koi at day 0 (non-infected) and 5 post-infection of CEV genogroup IIa. The gene expression was normalized to the expression of the gene encoding the S11 protein of the 40S subunit as a reference gene. Asterisks denote statistically significant differences marked with * at $p < 0.05$, with ** $p < 0.01$ between the control and infected once.

Full-size DOI: 10.7717/peerj.15614/fig-6

previous studies, it has been studied in a wide range of fish species (*Buonocore et al., 2003*; *Wang et al., 2006*). In our study, during both experiments (Genogroup I and Genogroup IIa infections), only koi carp showed up-regulation of IL-1β post-infection. This strong IL-1β inflammatory response may coincide with the previously conducted study where koi were found to be susceptible to both genogroups I or IIa infections (*Adamek et al., 2017c*). Furthermore, high expression of IL-1β was also found in different carp lines at

days 3 and 5 post-infection challenged with CyHV-3 (*Rakus et al., 2012*). IL-1β might be the significant driver of the gill pathology developing during the infection with CEV. This molecule was associated with the increased pathological changes in mucosal tissues during CyHV-3 vaccination studies (*Adamek et al., 2022a*) as well as tilapia lake virus (TiLV) susceptible strains of Nile tilapia (*Oreochromis niloticus*) (*Adamek et al., 2022b*). In both non-vaccinated carp which was subsequently challenged with CyHV-3 and TiLV susceptible strains of tilapia infected with the TiLV, the disruption of the skin and gill barriers and increased occlusion of the intralaminar spaces was associated with increased expression of the gene encoding for IL-1β. Current studies showing the increased IL-1β expression in the CEVD susceptible strains could explain the pathology recorded in these fish (*Adamek et al., 2017c*).

In our case, the low level of IL-1β in other strains might be due to the increased expression of IL-6a, which is a key immunosuppressive cytokine secreted by regulatory T-cells (*Zou & Secombes, 2016*). This is in accordance with the previous study where IL-6a significantly down-regulated the expression of IL-1β and TNF-α (key pro-inflammatory cytokines), suggesting its potential anti-inflammatory role in trout (*Costa et al., 2011*). In the current study, significant changes in the expression level of IL-6a were observed in AS, koi, and Rop strains infected with CEV genogroup I or IIa. Comparatively, the expression pattern was different between the genogroup I and IIa. Fish infected with genogroup I evidenced higher expression on days 6 and 11 compared to the control group. However, IL-6a was elevated on day 6 post-infection and declined on day 11 but remained higher than in the control. In common carp, the role of IL-6a in the response to viral infections is not defined yet. However, its crucial role in the humoral immune response was well observed in other fish species, such as Nile tilapia challenged with the bacterial infection which promotes IgM antibodies production (*Wei et al., 2018*). In fish, the expression of the IL-10 gene has also been linked to a decrease in inflammatory responses (*Forlenza, 2009*; *Ingerslev et al., 2009*; *Raida & Buchmann, 2008*). We observed relatively high down-regulation of the expression of IL-10 compared to the control group during the genogroup I infection on day 6 in koi carp. This low level was maintained till day 11 post-infection. On the contrary, the koi infected with genogroup IIa elevated the IL-10 expression on days 6 and 11 post-infection. IL-10 up-regulation to genogroup IIa could result from an increase in humoral immunity and inhibition of inflammation. Tumor necrosis factor (TNF) is a critical cytokine that plays an essential role in physiological and pathological processes. It promotes phagocytosis and nitric oxide production in teleost during viral and bacterial infections (*Tafalla, Figueras & Novoa, 2001*). Our results revealed higher expression of TNF-α2 in AS and Rop strains during both genogroups I and IIa infections. However, in koi, the significantly elevated level of TNF-α2 was only found on day 11 post-infection campared to the control group. The higher levels of TNF-α2 might be due to the elevation of IL-1β expression. This can be consistent with the previous study where IL-1β produced local effect on the expression of TNF-α in muscles that have been treated with a plasmid encoding IL-1β in japanese flounder *Paralichthys olivaceus* (*Taechavasonyoo, Hirono & Kondo, 2013*).

In the context of poxvirus infections, T helper cells play a critical role in the activation and differentiation of other immune cells, such as B cells and cytotoxic T cells (CTLs)

(*Yamaguchi et al., 2019*). CD4 and CD8 are proteins or cell surface markers found on the surface of T cells, a type of white blood cell that plays a critical role in the immune response (*Zamoyska, 1998*). CD4 is primarily found on T helper cells, which are a type of T cell that play an important role in coordinating the immune response by secreting cytokines and helping to activate other immune cells such as B cells and cytotoxic T cells (CTLs) (*Brown, Román & Swain, 2004*). Changes in the gene expression of CD4 and CD8 markers can provide important information about the state of the immune system and its response to infection or disease. For example, a decrease in CD4 expression may indicate a decrease in T helper cell function, which can impair the immune response and increase susceptibility to infections (*Adamek et al., 2021*). In mammals, the down-regulation of CD4 is the anchor that disarms actions against the immunity of several poxviruses. In our study, down-regulation of CD4 was observed in AS and koi strains during both genogroup I or IIa infections at days 6 and 11 post-infection compared to the control group. The highest down-regulation was observed in koi carp on days 6 and 11 post-infection to both genogroups. Similar, down-regulation of the gene encoding for the CD4 receptor of T-cells in koi under CEV infection was detected in gills on days 6 and 9 post-infection (*Adamek et al., 2021*). They suggest that possible reason for CD4 down-regulation in their study could be due to the immunosuppressive effect, which results from hyperammonemia in infected CEV carp. Since, in our findings, a strong down-regulation of CD4 in koi carp could be speculated to be caused by the elevated viral transcripts infected with genogroup I or IIa post-infection. In addition, we further analyzed the expression of CD8b1 along with *GzmA* (a protein-coding lysis gene produced in cytotoxic T-cells) to CEV infections in all four strains. CD8 is primarily found on cytotoxic T cells, which are responsible for recognizing and killing virus-infected cells (*Mosmann, Li & Sad, 1997*). According to the study of *Adamek et al. (2021)*, down-regulation expression of CD8b1 was noticed in the gills of CEV-infected koi carp compared to infected AS at any time points (day 0 to 13 post-infection). Interestingly, in our findings, among all groups only AS showed a tendency of down-regulation from day 6 to 11 in genogroups I or IIa infections compared to the control groups. In contrary, when carp lines were challenged with CyHV-3, up-regulation of CD8b1 was noted from day 1 to day 5 post-infection (*Rakus et al., 2012*). Poxviruses have been demonstrated to modulate the host immune system through various mechanisms. These mechanisms include the inhibition of immune cell activation and proliferation, downregulation of immune gene expression, and production of immunomodulatory proteins (*Howard et al., 1998*). For example, some poxviruses are able to suppress the expression of interferons, which play a critical role in initiating the host antiviral response (*Fensterl & Sen, 2009*). These viruses can also hinder the activation of natural killer cells and T cells, two essential components of the host immune system. Based on these findings, it is plausible to suggest that the suppressed expression of CD4 and CD8b1 in fish infected with carp edema virus may be also a result of viral immunomodulation of the host immune system. The *GzmA* levels were strongly down-regulated in koi at days 6 and 11 post-infection compared to the control to both CEV genogroups. The down-regulation of *GzmA* in AS infected with genogroup I was observed on days 6 and 11 post-infection. This down-regulation expression of *GzmA* could be due to the lower recruitment of

cytotoxic cells in gills due to CEV infection, which can also be seen in the case of CD4 and CD8b1 expression. Interestingly, down-regulation of cytotoxic cell markers such as *GzmA* in gills has been associated with cortisol-induced immunosuppression during salmon gill poxvirus (SGPV) infection (*Amundsen et al., 2021*). Based on SGPV and current CEV results, down-regulation of *GzmA* appears to be a very important marker that could predict the development of the acute form of the disease caused by fish poxviruses (*Amundsen et al., 2021*).

Several hundred genes are induced by type I interferon (IFN I), including some that encode direct antiviral effectors, such as the Mx proteins (*Fernández-Trujillo et al., 2015*). They can impede viral replication at different stages of the virus's life cycle (*Das et al., 2019*). The innate immune responses mediated by type I interferon against viruses and bacteria have been demonstrated in fish (*Langevin et al., 2013*). The crucial role of type I interferon-inducing genes in the fish immune response has been emphasized, including those that encode direct antiviral effectors (*Machat et al., 2021*). Moreover, interferon-induced genes are known to encode interferon-stimulated proteins, such as myxovirus resistance and protein kinase R, which exhibit direct antiviral activity. These proteins can effectively inhibit viral transcription, degrade viral RNA, inhibit translation, or modify the proteasome to control various stages of viral replication (*Sadler & Williams, 2008*). It was shown that different strains of common carp exhibit varying levels of IFN and ISG expression in response to viral infections, with susceptible fish showing a quicker response than resistant ones (*Machat et al., 2021*). As stated earlier, interferons (IFNs) and IFN-stimulated genes provide the first line of defense against viral infections. However, viruses have evolved strategies to escape immune surveillance and establish successful infections (*Rai et al., 2021*). Therefore, it is critical to understand the complex mechanisms of the interaction between viruses and the host's innate immune system, particularly the role of IFNs and IFN-stimulated genes, in developing effective treatment strategies for acute viral infections

The induction of type 1 interferon responses, the mRNA expression of the genes encoding interferon alpha-2 and interferon-induced proteins viperin and RNA dependent protein kinase, have been determined in the previously conducted infection trials from the same experimental study (*Adamek et al., 2017c*). Furthermore, in a very recent study conducted by *Adamek et al. (2021)*, koi and AS under CEV infections displayed up-regulation of type I IFN (*ifnα2*) expression in the gill and kidney compared to infected fish at any time points (day 0 to 13 post-infection).

Another important interferon regulatory protein, known as myxovirus resistance protein (Mx), mediate cellular resistance against wide range of viral pathogens (*Gao et al., 2011*). Therefore, we further confirmed the antiviral response of Mx2 during CEV genogroup I and IIa infections. When the carp strains were infected with CEV genogroup I, the mRNA expression of the Mx2 gene was significantly up-regulated in the gills of koi, PS and Rop at both time points days 6 and 11 p.i. compared to day 0. However, the peaked level of Mx2 was observed on day 6 in koi and PS strains, which proved to be susceptible to CEV genogroup I (*Adamek et al., 2017c*). The expression level of Mx2 was not observed in AS strain at any time points when compared to day 0. During the course of an infection with CEV

genogroup IIa, the mRNA expression of Mx2 was highly significantly up-regulated in koi at 6 and 11 days post-infection compared to control. Carp from other strains also showed the up-regulation of mRNA encoding Mx2 protein (particularly at day 6 post-infection), but the magnitude of up-regulation was significantly lower on day 11 post-infection. The evidence from the previous studies (*Adamek et al., 2014*; *Adamek et al., 2017c*) shows that the antiviral response seemed positively correlated with the viral load in infected fish and cannot be related to the resistance of carp strains to infection. In our findings, the same results were established in the case of Mx2 antiviral response, except of in PS strain, where Mx2 levels were not correlated to viral load and replication. Our findings, regarding the antiviral response of Mx2 correspond with above mentioned results where type I IFNs and interferon-induced proteins were significantly up-regulated in carp strains evaluated by *Adamek et al. (2017c)* and *Adamek et al. (2021)*.

In a primary antibody response, it is the IgM antibodies that constitute the majority of the body's antibodies (*Janeway Jr et al., 2001*). Unlike other types of antibodies, IgM are produced primarily by B1 cells, with no apparent stimulation by specific antigens (*Tumang et al., 2005*). Antigen-specific IgM is produced early following infection by most pathogens, followed by IgA, IgG, and IgE antibody responses (such as IgT/Z and IgD in fish) that are more specific (*Boes, 2000*). Like koi herpes virus disease (KHVD), the first CEVD mortality in carp occurs approximately a week after infection at a water temperature of 18–24 °C, and mortality may reach 100% at 11 days following infection (*Miyazaki, Isshiki & Katsuyuki, 2005*; *Piačková et al., 2013*; *Lewisch et al., 2015*). This indicates that the mortality occurs prior the beginning of antibody production or at a time when the level of antibodies is too low allowing the virus to replicate more freely in the carp body. What is more important, our study indicate significant down-regulation of IgM levels noticed in koi on day 11 post-infection to CEV genogroups I or IIa compared to the control group which is an accordance with the findings of *Adamek et al. (2021)*, who reported the down-regulation expression of IgM in the gills of CEV infected koi with two-fold at day 3 and five-fold at day 9 p.i.. This could suggest that CEV infection process is delaying the start of antibody response however this requires further studies. Especially due to the fact, there were only slight variations in IgM levels in other carp strains but not significantly different.

The significant level of mucin conservation across all vertebrates serves to underscore their critical role in defending against pathogen intrusion. The pivotal function of mucin in the mucosal barrier of fish was demonstrated by the linkage of single nucleotide polymorphisms in mucin 2 and 5b with the resistance of rohu carp (*Labeo rohita*) to Aeromonas hydrophila infection (*Robinson et al., 2014*), thus underscoring the significance of mucin in host-pathogen interactions. In common carp, mucin 2 and mucin 5b expression was found to increase during beta glucan feeding (*Van der Marel et al., 2012*), while it decreased during CyHV-3 infection (*Adamek et al., 2013*), highlighting the versatility of mucin as a biomarker in different physiological settings. The present study investigated the impact of CEV genogroup I or IIa exposure on Muc5b gene expression in the gills of four distinct fish strains. The findings indicated that there was no substantial difference in the Muc5b gene expression on days 6 and 11 p.i. in the gills of all four strains compared to the control. Nonetheless, in the gills of koi carp, a negligible and non-significant decrease

in the Muc5b mRNA level was observed on day 11 after the exposure to CEV genogroup I or IIa, which was marginally different from the other fish strains. Our study differed from a previous investigation on common carp, which revealed that MUC5 gene expression in the fish gills was significantly up-regulated following dietary beta-glucan administration (*Van der Marel et al., 2012*). In zebrafish (*Danio rerio*), Similarly, administration of pectin in the diet resulted in a significant up-regulation of whole body MUC5 gene expression, as reported in a recent study (*Edirisinghe et al., 2019*). To date, the expression of other mucins in CEV-infected fish has only been investigated by *Adamek et al. (2017a)*. Their study reported a decrease in muc2-like transcripts in common carp at 144 h post-infection. In contrast, our study did not observe any significant changes in muc5b expression levels in CEV-infected carp gills at both 6 and 11 days post-infection. Hence, the potential role of muc5b in the susceptibility of CEV-infected strains to both genogroups of the virus remains unclear.

Immune gene responses in mucosal tissues to CEV infection

In fish, the thymus and head kidney serve as the primary lymphoid organs, whereas the spleen, trunk kidney, and mucosa-associated lymphoid tissue (MALT) located in areas like the skin, gills, intestine, oral and nasal mucosa, and urogenital tract comprise the secondary lymphoid organs (*Ángeles Esteban, 2012*; *Soulliere & Dixon, 2017*). The MALT can be further divided by anatomical location into skin-associated lymphoid tissue (SALT), gut-associated lymphoid tissue (GALT), and gill-associated lymphoid tissue (GIALT) (*Ángeles Esteban, 2012*; *Salinas, Zhang & Sunyer, 2011*).

Skin, gills, and intestines of fish are the first barriers with the biggest mucosal surface providing the interface between a fish and its environment. As a defence mechanism against invading pathogens, these tissues secrete antimicrobial humoral factors, which act through various mechanisms to limit the spread and proliferation of pathogens (*Mehana, Rahmani & Aly, 2015*). In this study, AS and koi carps were exposed to CEV genogroup IIa to determine the immune gene expression in these mucosal tissues. Interestingly, the results indicate that not only the gills as a target tissue for the virus but also other mucosa react to the infection. Namely, the mRNA expression level of selected cytokine genes were mainly regulated in koi carp's gill and gut post-CEV infection. The detection of changes in expression of innate immune genes, such as IL-10, IL-1β, TNF-α2 and IL-6a, during CEV infection suggests that innate immunity in these mucosal tissues played a significant role in the antiviral process. Similarly, in a very recent study conducted by *Kushala et al. (2022)*, significant up-regulation of IL-10, IL-1β, and TNF-α were detected in the gill of naturally KSD-affected koi. Interestingly, it was found that mucosal immunity plays even more important role in protection when a cohabitation method is used for inducing the infection. For instance, when different Nile tilapia strains were infected with tilapia lake virus (TiLV), the virus load was significantly lower with less mortality in the strains infected through the cohabitation method compared to strains infected with an intraperitoneal injection (*Adamek et al., 2022b*). In our case, despite gill containing the highest virus load, the strong innate immune mucosal response could be seen in gill and gut tissues in carp strains infected through the cohabitation method.

The common carp's ability to defend itself against viral infections is underlined by the overproduction of mucus on skin and gill during CEV infection (*Zhang et al., 2017*). And it has been recorded that skin epidermal erosion following partial ulceration is one of the main sign during CEV infections (*Miyazaki, Isshiki & Katsuyuki, 2005*). In our case, skin despite showing the second highest viral loads of CEV, did not induce strong innate immune response, except IL-1β, the only gene that was up-regulated in koi carp during post-CEV infection. It seems that other immune genes related to skin protection could play a significant role during the infection with this pathogen. For instance, mucins (a membrane-associated glycoprotein) that are the main components of the mucosal barrier studied during the infections with carp viruses CyHV-3, and SVCV including CEV (genogroup I) (*Adamek et al., 2017a*). The down-regulation of mucin mRNA expression was detected in gill and gut mucosal tissues of carp infected with above cited pathogens.

There is a far paucity of research about T cell markers (CD4, CD8b1 and *GzmA*) to CEV infection in the mucosal tissue of common carp. Gill is the only mucosal organ where down-regulation of gene encoding for CD4 and CD8 cytotoxic T cells markers were detected in koi on day 6 and 9 post-exposure to CEV genogroup IIa infection compared with control as well to the expression in CEV-infected AS carp (*Adamek et al., 2021*). Our findings agree with their study reporting down-regulation of T cell markers such as CD4 and *GzmA* in koi gill on 5 dpi to CEV genogroup IIa compared to control and AS strains. Further, no CD4, CD8b1 and *GzmA* responses were noticed in other mucosal organs, such as gut and skin. Interestingly up-regulation of Mx2 was noticed in all mucosal organs during CEV exposure indicating that mucosal tissues induced a robust antiviral response to CEV infection. The evidence from the previous studies (*Adamek et al., 2014*; *Adamek et al., 2017c*) shows that the antiviral response seemed positively correlated with the virus load in infected fish and cannot be related to the resistance of carp strains to infection. Our study found a similar correlation between virus load and antiviral response in gill tissue. However, the gut harboured the least amount of virus load among the mucosal organs, showed similar up-regulation expression levels of Mx2 in both strains during post-CEV exposure.

Furthermore, mucosal response of mucin-encoding genes (Muc2 and Muc5b) in AS and koi upon CEV genogroup IIa infection in gill, gut and skin tissues was evaluated. In our case tissue-specific expression of mucin genes was observed, where Muc2 was expressed exclusively in the gut tissue, and Muc5b was expressed in the gill and skin tissues. Tissue specific expression of Muc2 and Muc5b genes have been noticed in the previous studies. For instance, Muc2 in carp is predominantly expressed in gut and Muc5b gene, mostly expressed in skin and brancial epithelium (*Van der Marel et al., 2012*). In addition, the gills predominantly express Muc2-like as the secreted mucin when compared to Muc5b, while the skin mainly secretes Muc5b in comparison to Muc2-like (*Lang et al., 2004*). Muc2-like, Muc5b, and the previously described Muc2 from the gut are the primary secreted mucins in the major mucosal tissues of the common carp. The current study revealed a marked down-regulation of Muc5b transcripts in the skin tissue of koi at day 5 post-infection as compared to the control group. These findings suggest that Muc5b may play a critical role in the defense mechanisms of koi against the infectious agent. The reduced expression of

Muc5b transcripts in the skin tissue may lead to impaired mucus production, which may affect the skin barrier function and increase the susceptibility of koi to infection.

CONCLUSIONS

In conclusion, this is the first study where important immune gene expression was determined in different carp strains infected with CEV genogroup I and compared with responses against genogroup IIa. Fish infected with both genogroups demonstrated similar expression patterns of selected immune-related genes. Interestingly, koi carp was the only strain where most genes showed significant differences in fish infected with both CEV genogroups, with slight variation in the expression pattern. Furthermore, the expression pattern for most genes in KSD-resistant AS strain (*Adamek et al., 2017c*; *Adamek et al., 2021*) resemble some similarities to koi. These similarities might be due to the origin of both carp strains from the same species, such as *Cyprinus rubrofuscus*. According to the observed expression patterns, the difference in susceptibility does not seem to be related to the kinetics of expression of selected immune genes studied in this work. The expression patterns however could help explaining the recorded pathology. Furthermore, up-regulation of mRNA expression of most of the selected immune genes in koi gill and gut tissues suggests potential systemic mucosal response against CEV infection. Ultimately, the implementation of further studies of immune responses against CEV should be under strong consideration because of the paucity of literature regarding the immune responses of carp to CEV infections.

ADDITIONAL INFORMATION AND DECLARATIONS

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Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Ali Asghar Baloch performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the article, and approved the final draft.

- Dieter Steinhagen conceived and designed the experiments, analyzed the data, authored or reviewed drafts of the article, and approved the final draft.
- David Gela conceived and designed the experiments, performed the experiments, authored or reviewed drafts of the article, and approved the final draft.
- Martin Kocour conceived and designed the experiments, performed the experiments, authored or reviewed drafts of the article, and approved the final draft.
- Veronika Piačková conceived and designed the experiments, performed the experiments, analyzed the data, authored or reviewed drafts of the article, and approved the final draft.
- Mikolaj Adamek conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the article, and approved the final draft.

Animal Ethics

The following information was supplied relating to ethical approvals (*i.e.*, approving body and any reference numbers):

Lower Saxony State Office for Consumer Protection and Food Safety (LAVES), Oldenburg, Germany prodided full approval for this research under the reference number: 33.19–425 2-04-16/2144.

Data Availability

The following information was supplied regarding data availability:

The raw data is available in the Supplemental Files.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.15614#supplemental-information.

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CHAPTER 4

General discussion

Fish, being aquatic animals, have a close interface with their environment – water, and they are fully dependent on this. As the water in which fish live is never only H_2O but it contains a lot of solved and insoluble matters and lives organisms, its composition is very variable. Except for others, a diverse range of microorganisms is present, some of which may be pathogenic. The more intensive the fish farming, the more fragile the balance between the individual components and the higher risk of infection and transmission of infectious diseases (Road, 1999). The worldwide expansion of fish aquaculture and the efforts to reduce drug consumption require improvement of zoohygiene in fish breeds, and the importance of prophylaxis and prevention measures to mitigate the prevalence of fish diseases come to the fore. The production of carp has particularly experienced a surge, leading to an increase in the incidence of several types of diseases. Therefore, there is an emerging necessity to conduct research on carp diseases. Although changes in the physicochemical characteristics of water may impact the well-being of fish and generate severe issues, the main reason for mortality in these animals is the proliferation of diverse microorganisms that result in various types of carp diseases. Unlike bacterial diseases, where antibiotics can be used in extreme cases, no targeted treatment for viral diseases is available. Fish must therefore cope with exposure to pathogens by using their own immune mechanisms. Therefore, the only way to increase the fish's chances of survival is to promote their natural resistance to infectious agents and increase their tolerance to negative environmental influences. This requires a thorough understanding of the mechanisms of defense reactions of fish immune system. The thesis offers a detailed overview of the immune response of common carp of various strains, revealing crucial information about the carp's immune system and its ability to respond to different infections.

The second chapter of the thesis presents a review of cytokine responses in common carp subjected to diverse diseases, as discussed in Baloch et al. (2022). The principal objective of this study was to provide a comprehensive synthesis of the existing literature on immune responses, particularly cytokine expression, in common carp exposed to important diseases. Our overview highlights the crucial role of cytokines, which function as inflammatory mediators, and their mechanisms in response to pathogenic and non-pathogenic immunostimulants in fish. The production of cytokines leads to the recruitment of inflammatory cells that actively participate in the clearance of diverse infections which may manifest in various symptoms and signs of infections.

Fish rely heavily on the innate immune system for immune defense, which includes cytokines and cytokine-producing cells that act as essential components of the immune response against various infections. This small signaling proteins secreted by immune cells such as T lymphocytes, B lymphocytes, mast cells, and endothelial cells are responsible for regulation of the immune response (Tortorella et al., 2000). Cytokines trigger non-specific immune responses, induce cytotoxic T cells, and production of antibodies, and regulate the production of cytokines by B cells, as stated by Kerkeni et al. (2016). Cytokines are classified based on their structural characteristics as interferons (IFN), interleukins (IL), tumor necrosis factors (TNF), transforming growth factors (TGFs), and chemokines (Alejo et al., 2011). Various infectious stimuli can trigger the production of cytokines, making host-derived cytokines effective tools for investigating immune responses in the early stages of viral and bacterial infections. In particular, interferons, interleukins, and tumor necrosis factors have been found to play a significant role in clearing pathogens and enhancing antimicrobial activity in carp, as highlighted by a review article.

Carp edema virus disease first appeared in European fish farms approximately ten years ago and exhibits symptoms akin to those of KHV, including gill necrosis, sunken eyes, irregular skin-mucus production, and mortality, as reported by Way et al. (2017). However, compared to KHVD, the disease occurs more frequently at lower water temperatures. The disease may have originated from the virus that causes " koi sleepy disease," first detected in Japanese koi farms during the 1970s (Way and Stone, 2013). Matras et al. (2017) identified several different genogroups of this viral pathogen through recent molecular biology research. Although the disease has similar spread and economic impact to KHVD, it has gone unnoticed, and its prevalence remains unknown. The OIE considers it to be an emerging disease. Upon closer examination of the literature concerning carp edema virus (CEV) infection in carp and immune responses to combat them, some gaps and limitations were identified. These gaps may be attributed to the historical fact that poxviruses alter the host's immune response by encoding various immunomodulatory proteins that can inhibit host antiviral responses to ensure their survival (Howard et al., 1998). Additionally, the absence of a standardized procedure for isolating and amplifying CEV in cell lines has impeded progress in research related to the immune response to CEV infections. The identification of numerous emerging genogroups of CEV in carp populations worldwide has left the virus without a definitive classification. Thus, when carp are infected with CEV, the interaction between the host and virus is complex and influenced by both host genetic competitiveness and viral genomic features. A study showed that strains of carp greatly impact the cytokine expression profile during infections, especially interferons (IFNs) and interferon-stimulated genes (ISGs) (Adamek et al., 2017a). However, IFNs were not found to be linked to the resistance or susceptibility of CEV-affected carp strains. Susceptible carp strains infected with CyHV-3 or SVCV demonstrated a rapid upregulation of IFNs and ISGs, unlike resistant strains (Adamek et al., 2019; Tadmor et al., 2019). In addition, a recent study found significant up-regulation of IL-10, IL-1β, and TNF-α in the gill of naturally KSD-affected koi (Kushala et al., 2022). Compared to CyHV-3 and SVCV infections, research on immune gene responses during CEV infection in common carp is somewhat scarce, indicating the need for further studies to better understand carp immune responses to CEV infections.

In the same chapter, we further outlined the cytokine responses of common carp to infections caused by Cyprinid herpesvirus 3 (CyHV-3), also known as koi herpes virus (KHV). The emergence of KHVD dates to back to the late 1990s when it was first spread in Israel, primarily due to the international trade of ornamental fish (Hedrick et al., 2000). Considering its infectious, fatal, and financially noteworthy properties, the European Council (2006/88/EC) has designated it as a non-exotic disease and appended it to Annex II of the Animal Health Law (Regulation (EU) 2016/429). The disease is also listed by the OIE (World Organization for Animal Health). This part of the chapter presents a comprehensive review of existing studies on gene expression analysis of immune-related genes in common carp in response to KHV infection. In humans and other vertebrates, the non-specific immune response to herpesviruses primarily involves the activation of natural killer (NK) cells and the production of interferons and other cytokines (Mossman et al., 2005). The role of interferon (IFN) in innate immunity is crucial in defending the body against viral invasion. This cytokine contributes significantly to the early control of herpesvirus infections due to its immunoreactive properties, leading to several type 1 IFN responses, including ISGs (Adamek et al., 2014b; Kitao et al., 2009). Following a comprehensive analysis of the literature, we determined that interferons (IFNs) and/or interferon-stimulated genes (ISGs) were found to be significant regulators of inflammation during Cyprinid herpesvirus 3 (CyHV-3) infections in common carp (Adamek et al., 2012, 2019). Upregulation of IFNs and/or ISGs was predominantly observed in common carp during CyHV-3 infections (Baloch et al., 2022). While the role of specific cytokines in CyHV-3 infection is not fully understood, the virus triggers a cell-mediated

immune response by producing specific immunoglobulins (Ig) (Forlenza et al., 2009). In the late stages of herpesvirus infection, adaptive immunity is activated by enhancing antibody production through TNF-β and IFN-γ, leading to stimulation of natural killer T cells and B lymphocytes. Despite this, all known herpesviruses have the capacity to develop persistent latent infections. The virus either mimics host immune responses to manage those latent infections or encodes its own antiviral-like proteins (Davison et al., 2009; Beurden et al., 2011). Sunarto et al. (2012) reported that CyHV-3 hijacks an IL-10 gene from the host and modifies its features to deal with the host's immune response. However, in-vitro studies by Ouyang et al. (2013) demonstrated that CyHV-3 ORF134, which encodes an IL-10 homolog, is neither necessary nor sufficient for viral replication or virulence in common carp.

Building on the topics covered in the preceding chapter, we also reviewed how fish cytokines respond to SVC infection. The host range of SVCV is broader than that of KHV and CEV, as it infects multiple species of fish, including members of the Cyprinidae family (Ahne et al., 2002). Despite its minimal impact on carp farming economics, the disease remains a focus of OIE monitoring due to its presence in various regions worldwide. SVCV has been identified in Europe, North America, South America, and Asia, although the actual extent of global spread is uncertain, given the lack of systematic surveillance. Following a comprehensive analysis of the literature, we determined that the immune system of fish infected with rhabdoviruses is regulated by group I and II interferons (IFNs). The antiviral effects of group I IFNs have been shown to inhibit replication at all stages of the virus (Wang et al., 2006; Zou et al., 2007; Chaved-Pozo et al., 2010;). In contrast to group I IFNs response, the mechanism by which group II IFNs control rhabdoviruses is more varied (Zou et al., 2007). By increasing the expression of interferon regulatory genes such as myxovirus resistance protein (Mx) and viperin, recombinant IFNs-II have been shown to induce a protective effect against SVCV infection in zebrafish. Conversely, group 1 IFNs demonstrate more sustained induction of both ISGs and pro-inflammatory cytokines (IL-10β and TNF-α) (López-Muñoz et al., 2009). Research conducted in vitro and in vivo with carp indicates that IFNs-I have alternative responses during SVCV and CyHV-3 infections. In a study using CCB cells infected with either SVCV or CyHV-3, a higher upregulation of IFN-I was found with SVCV, while CyHV-3 conversely suppressed IFN-I production (Adamek et al., 2012). A comparable IFN response pattern was identified in different common carp strains (Amur wild carp, Amur sasan, AS; Ropsha scaly carp, Rop; Prerov scaly carp, PS; and koi) when they were exposed to the same viruses (Adamek et al., 2019). The Rop strain showed more resistance against SVCV infection than the PS strain, whereas both Rop and AS strains exhibited improved survival rates during CyHV-3 infection when compared to the PS strain. The differential response of carp to SVCV and CyHV-3 infections seems to be associated with the increased activity of IFN-I among the SVCV-resistant group, as compared to the CyHV-3 resistant group. These findings indicate that the contribution of type 1 interferon in preventing SVCV infection is specific to the host-pathogen interaction with the SVC virus, and not with CyHV-3.

The final segment of this chapter comprises a discussion of the immune responses evoked by Aeromonas hydrophila pathogen in common carp. Aeromonas hydrophila, a Gram-negative rod-shaped bacterium, is among the most prevalent bacterial pathogens that infect freshwater fish (Vivekanandhan et al., 2002). Studies have demonstrated that Aeromonas hydrophila bacteria naturally reside in the gastrointestinal tracts of fish (Tanekhy et al., 2009). Common carp, catfish, goldfish, and other tropical and ornamental fish, which are highly susceptible to Aeromonas hydrophila infection, are mainly affected by this bacterium (Brum et al., 2017). The health of carp in intensive farming systems is frequently jeopardized by Aeromonas hydrophila infection, which is the causative agent of bacterial hemorrhagic septicaemia or motile aeromonad disease. As a result, the aquaculture industry often suffers from substantial

economic losses (Nielsen et al., 2001). The association of *Aeromonas hydrophila* with cytokine responses in common carp, which is extensively studied, is reflected in this review by emphasizing the immunoreactivity based on cytokines elicited by this pathogen. In the wake of a careful scrutiny of the literature, we observed that during *A. hydrophila* infections, the secretion of cytokines such as ILs, IFNs, and TNFs is of great importance. Of these, interleukins appear to be the focal point of immunological research interest, followed by tumor necrosis factors and interferons. IL1-β, IL-10, and IFN expression in carp during *A. hydrophila* infection are believed to be primarily triggered by toll-like receptor 18 (Tlr18), as suggested by a study on *Epithelioma papulosum cyprini* cell lines (EPC) (Shan et al., 2018). Additionally, numerous investigations have been carried out to assess the IL-1β response during *A. hydrophila* infections. For instance, carp exposed to high temperature (30 °C) for 30 days and subsequently challenged with *A. hydrophila* displayed significantly increased IL-1β and TNFα expression in the spleen and head kidney (Shahi et al., 2018). In addition, we discovered the involvement of IL-17 family ligands in fish in response to *A. hydrophila* infection. These ligands have tissue-specific roles, with expression being abundant in mucosal tissues, such as the gills, skin, and intestine (Kono et al., 2011). The gills of common carp exhibited active expression of genes belonging to the IL-17 family in response to *A. hydrophila* (Dong et al., 2019). Upregulation of IL-17 genes occurs in the gills of common carp during *A. hydrophila* infection, with IL17A/F2 and IL17N showing the most prolonged upregulation (Kono et al., 2011). Recently, common carp interleukins (IL-17Na and b) were identified and confirmed in response to *A. hydrophila* infection (Li et al., 2021). These genes play a pivotal role in regulating early immune responses in fish. The available data indicate that interferon regulatory factors (IRFs) are important in regulating the innate immune response in fish (Zhu et al., 2020). For instance, carp challenged with *A. hydrophila* and poly I:C showed increased expression of IRF9, which has antiviral and antibacterial properties (Zhu et al., 2019). Meanwhile, IRF10 helps stabilize bacterial infection by inhibiting IFN response, possibly to prevent excessive immunopathological reactions and secondary viral infections (Zhu et al., 2020). Collectively, the interferon regulatory factors (IRFs) exert a crucial influence on the regulation of type 1 interferon (IFN) and interferon-stimulated gene (ISG) expression in common carp subjected to *A. hydrophila* infection. Investigating the extent and potency of IRF gene expression in common could be a promising avenue for future research.

The third chapter of the thesis endeavored to evaluate the immune responses of different strains of carp fish with varying susceptibilities to carp edema virus (CEV). Various common carp strains with distinct genetic backgrounds may exhibit significant differences in their susceptibility to cyprinid herpesvirus 3 (CyHV-3) infection, as previous research has indicated (Shapira et al., 2005; Rakus et al., 2009; Piačková et al., 2013; Adamek et al., 2019). Similarly, immune-related gene expression patterns have been widely studied in many viral diseases in fish, research into these patterns in carp strains infected with CEV has been limited in comparison. Historical evidence suggests that poxviruses modulate host immune system through various mechanisms including immunomodulatory proteins, which can hinder the host's antiviral responses and ensure the virus's survival. These mechanisms include ways to slow down the activation and growth of immune cells, decrease the expression of immune genes, and produce proteins that can change how the immune system responds (Howard et al., 1998). For instance, poxviruses can interfere with the body's production of interferons, which are key players in the immune system's response to viral infections (Fensterl and Sen, 2009).

Our in-depth analysis of the current literature on immune responses, with a particular focus on cytokine expression, in common carp exposed to key diseases, has also revealed that compared to other viral diseases in fish, there has been a scarcity of research conducted on the

gene expression patterns of immune-related genes in carp strains infected with CEV. Therefore, the present study assesses the immune gene responses in the gills of various carp strains, including AS, koi, PS, and Rop, upon infection with CEV genogroups I and IIa. Furthermore, to assess the capacity of mucosal immune genes to respond to CEV infection, this study also subjected two carp strains (KSD-susceptible koi and KSD-resistant AS) to CEV genogroup IIa. A total of eleven immune-related genes in the gills of four different carp strains with varying susceptibility to KSD. These genes encoded cytokines (IL-1β, IL-10, IL-6a, and TNF-α2), antiviral response (Mx2), cellular receptors (CD4, CD8b1, and GzmA), immunoglobulin (IgM), and mucins (Muc5b and Muc2). The expression patterns of these genes were monitored in gills of strains on days 6 and 11 post-infection to both CEV genogroups (I and IIa) in experiment part one and in gill, gut, and skin tissues on day 5 post-infection to genogroup IIa in experiment part two.

In the current chapter, the expressions of cytokine genes, namely IL-1β, IL-6a, IL-10, and TNF-α2, in different carp strains, AS, PS, Rop and koi, on days 6 and 11 post carp adema virus infection experiments for Genogroup I and Genogroup IIa infections. The study found that koi carp showed up-regulation of IL-1β post-infection during both experiments, which coincided with the previous study's observation of koi's susceptibility to both genogroups I or IIa infections (Adamek et al., 2017a). Additionally, high expression of IL-1β was found in different carp lines at days 3 and 5 post-infection challenged with CyHV-3 (Rakus et al., 2012). Significant changes in the expression level of IL-6a were observed in AS, koi, and Rop strains infected with CEV genogroup I or IIa, and fish infected with genogroup I evidenced higher expression on days 6 and 11 compared to the control group. On the contrary, the expression pattern was different between the genogroup I and IIa, and IL-6a was elevated but remained higher than in the control. In common carp, the role of IL-6a in the response to viral infections is not defined yet. Its crucial role in the humoral immune response was well observed in other fish species, such as Nile tilapia challenged with the bacterial infection which promotes IgM antibodies production (Wei et al., 2018). The expression of the IL-10 gene in koi carp was down-regulated compared to the control group during the genogroup I infection on day 6, while IL-10 up-regulation was observed in koi infected with genogroup IIa. Moreover, the study revealed higher expression of TNF-α2 in AS and Rop strains during both genogroups I and IIa infections, and in koi, the significantly elevated level of TNF-α2 was only found on day 11 post-infection compared to the control group. The higher levels of TNF-α2 might be due to the elevation of IL-1β expression. This can be consistent with the previous study where IL-1β produced local effect on the expression of TNF-α in muscles that have been treated with a plasmid encoding IL-1β in Japanese flounder Paralichthys olivaceus (Taechavasonyoo et al., 2013). In fish, the expression of the IL-10 gene has also been linked to a decrease in inflammatory responses (Raida and Buchmann, 2008; Forlenza, 2009; Ingerslev et al., 2009). Furthermore, in order to investigate the similar cytokine gene expression in mucosal tissues, AS and koi carps were subjected to CEV genogroup IIa. Surprisingly, the mRNA expression of all cytokine genes was observed to be predominantly up-regulated in the gill and gut of koi carps following CEV infection.

The involvement of T helper cells in the activation and differentiation of immune cells during poxvirus infections is crucial (Yamaguchi et al., 2019). T cells are white blood cells that play a critical role in the immune response and are characterized by the presence of CD4 and CD8 proteins or cell surface markers (Zamoyska, 1998). In our study, down-regulation of CD4 was observed in AS and koi strains during both genogroup I or IIa infections at days 6 and 11 post-infection compared to the control group. The highest down-regulation was observed in koi carp on days 6 and 11 post-infection to both genogroups. Additionally, Adamek et al. (2021) detected down-regulation of the gene encoding for the CD4 receptor of T-cells in koi gills on

days 6 and 9 post-infection under CEV infection. We also analyzed the expression of CD8b1 and GzmA in response to CEV infections in all four strains. Adamek et al. (2021) reported down-regulation of CD8b1 in the gills of CEV-infected koi carp compared to infected AS at any time points (day 0 to 13 post-infection). Interestingly, our findings showed that only AS had a tendency for down-regulation of CD8b1 from day 6 to 11 in genogroups I or IIa infections compared to the control groups. However, up-regulation of CD8b1 was noted from day 1 to day 5 post-infection when carp lines were challenged with CyHV-3 (Rakus et al., 2012). GzmA levels were strongly down-regulated in koi at days 6 and 11 post-infection compared to the control for both CEV genogroups, and down-regulation of GzmA in AS infected with genogroup I was observed on days 6 and 11 post-infection. This down-regulation of cytotoxic cell markers could be due to the lower recruitment of cytotoxic cells in gills due to CEV infection, which is consistent with the down-regulation of CD4 and CD8b1 expression. Similarly, down-regulation of cytotoxic cell markers such as GzmA in gills has been associated with cortisol-induced immunosuppression during salmon gill poxvirus (SGPV) infection (Amundsen et al., 2021). Additionally, T cell markers (CD4, CD8b1, and *GzmA*) were analyzed in the mucosal tissue of AS and koi in response to CEV infection. Down-regulation detection of the gene encoding for CD4 and CD8 cytotoxic T cell markers in koi gills on day 6 and 9 post-exposure to CEV genogroup IIa infection compared with control as well as to the expression in CEV-infected AS carp (Adamek et al., 2021). Our findings agree with their study, reporting down-regulation of T cell markers such as CD4 and GzmA in koi gill on 5 d.p.i to CEV genogroup IIa compared to control and AS strains. Finally, no CD4, CD8b1, and *GzmA* responses were noticed in other mucosal organs, such as gut and skin.

According to Sadler and Williams (2008), interferon-induced genes encode interferon-stimulated proteins such as myxovirus resistance and protein kinase R that directly exhibit antiviral activity. These proteins can inhibit viral transcription, degrade viral RNA, inhibit translation, or modify the proteasome to control different stages of viral replication. In our study, we confirmed the antiviral response of Mx2 during CEV genogroup I and IIa infections. Adamek et al. (2017a) reported that the mRNA expression of the Mx2 gene was significantly up-regulated in the gills of koi, PS, and Rop at both time points (days 6 and 11 p.i) compared to day 0 during CEV genogroup IIa infection. However, the peaked level of Mx2 was observed on day 6 in koi and PS strains, which were found to be susceptible to CEV genogroup I. In contrast, the expression level of Mx2 was not observed in AS strain at any time points when compared to day 0. However, during genogroup I infection, up-regulation of Mx2 was detected in koi and AS. Interestingly, up-regulation of Mx2 was noticed in all mucosal organs during CEV exposure, indicating that mucosal tissues induced a robust antiviral response to CEV infection. Furthermore, previous studies have shown that the antiviral response is positively correlated with the virus load in infected fish and cannot be related to the resistance of carp strains to infection (Adamek et al., 2014a; 2017a). Our study found a similar correlation between virus load and antiviral response in gill tissue. However, the gut, which had the least amount of virus load among the mucosal organs, showed similar up-regulation expression levels of Mx2 in both strains during post-CEV exposure.

Similar to koi herpes virus disease (KHVD), the initial mortality in carp during CEVD typically transpires around one week after infection at a water temperature of 18–24 °C, and the mortality rate may escalate to 100% by day 11 post-infection (Miyazaki et al., 2005; Piačková et al., 2013; Lewisch et al., 2015). These observations suggest that mortality in carp occurs either prior to the commencement of antibody production or during a phase when the antibody level is inadequate, thereby enabling the virus to propagate more unreservedly within the carp organism. Our study demonstrated a significant decrease in the levels of IgM in koi on the 11th day post-infection with CEV genogroups I or IIa when compared to the control group. This

finding is consistent with the results reported by Adamek et al. (2021) who observed a two-fold and five-fold decrease in the expression of IgM in the gills of CEV-infected koi on day 3 and day 9 post-infection, respectively. These observations suggest that the process of CEV infection may delay the onset of the antibody response. However, additional investigations are required to verify this hypothesis. Interestingly, when IgM was analysed in gill, gut, and skin tissues, only skin showed the down-regulated expression on day 5th post CEV infection.

The significance of mucin in host-pathogen interactions in fish was demonstrated through the linkage of single nucleotide polymorphisms in mucin 2 and 5b with the resistance of rohu carp (*Labeo rohita*) to *Aeromonas hydrophila* infection, as reported by Robinson et al. (2014). In the present study, there was no significant difference in the expression of the Muc5b gene in the gills of all four strains compared to the control on days 6 and 11 post-infection (p.i). However, a slight and non-significant decrease in the Muc5b mRNA level on day 11 after exposure to CEV genogroup I or IIa in the gills of koi carp was observed, which was marginally different from the other fish strains. This contrasts with a previous investigation on common carp, which revealed that MUC5 gene expression in the fish gills was significantly up-regulated after dietary beta-glucan administration, as reported by van der Marel et al. (2012). Adamek et al. (2017b) investigated the expression of other mucins in CEV-infected fish and reported a decrease in muc2-like transcripts in common carp at 144 hours post-infection. However, our research did not identify any noteworthy alterations in muc5b expression levels in the gills of CEV-infected carp at either 6 or 11 days p.i. These findings suggest that the role of Muc5b in the immune response to CEV infection may be strain-specific and that further studies are required to elucidate its role in the pathogenesis of CEV infection in fish.

As an unpublished result, the experiment described here represents a short trial conducted in our laboratory. In the present study, we examined the prevalence of CEV infection in various fish strains, including koi, Madar, Madar x Al, Rop x TAT, and AS strains. Several fish from all strains were found to be infected with CEV, except for AS strains where only a single fish was CEV positive. Among the infected fish, koi carp exhibited noticeable behavioral changes and clinical signs, suggesting a more pronounced impact of CEV infection on this particular species. The main purpose of conducting this short trial was to determine whether lysozyme levels in CEV-infected strains are enhanced or not. Lysozyme plays a critical role as a defense molecule within the innate immune system, serving as a crucial mediator in safeguarding against microbial invasions (Saurabh and Sahoo, 2008). The level or activity of lysozyme is widely recognized as a significant indicator of the innate immunity of fish and is found ubiquitously across various living organisms. It has been employed as a marker for assessing the response to aquatic stress and the capacity for disease resistance (Saurabh and Sahoo, 2008). Despite the recognized significance of lysozyme in defending against infectious diseases in fish, limited research has been conducted on lysozymes specific to common carp species. In our study, despite the observed changes in koi carp, we did not find a significant difference in lysozyme activity compared to uninfected individuals. Our results agree with the published findings (Papežíková et al., 2023), where no significant difference in lysozyme activity was found in the mucus of CEV-infected fish. However, when serum lysozyme activity was evaluated in various fish species such as common carp (*Cyprinus carpio*), pikeperch (*Sander lucioperca*), prussian carp (*Carassius gibelio*), and crayfish (*Astacus leptodactylus*), common carp was found to exhibit higher levels of lysozyme, being the second highest. Furthermore, when common carp were fed with immunomodulators such as chitin, chitosan, and levamisole for 90 days and then challenged with *Aeromonas hydrophila*, lysozyme, along with other immune parameters including white blood cell count (WBC) and respiratory burst activity (NBT assay), were significantly stimulated (Gopalakannan and Arul, 2006). Interestingly, lysozyme activities in Madar and Madar x Al strains were found to have significantly higher levels compared

to uninfected fish. This suggests that these fish strains may have an enhanced lysozyme-mediated immune response against CEV.

To validate these results further and gain a more comprehensive understanding, controlled experiments could be conducted to determine lysozyme activity alongside other immune parameters. Assessing additional factors such as white blood cell count (WBC) and respiratory burst activity (NBT assay) would provide a more comprehensive evaluation of the immune response. These control experiments would help to strengthen the validity of the observed findings and deepen our knowledge of lysozyme-mediated immune responses in CEV-infected fish.

Conclusions

The dissertation thesis submits results of the immune response displayed by common carp and/or strains against a range of diseases, exposing fundamental information concerning the immune system of carp and its capacity to counter various infections.

The following conclusions can be drawn from the review article:

- Cytokines are crucial in fish's immune response to pathogens as they trigger the recruitment of inflammatory cells and help eradicate infections.
- Different types of cytokines are generated in common carp based on the type of infectious agent.
- Variability in cytokine responses is caused by the disease itself, as evidenced by variations in interferon and interleukin generation in different infections.
- Studying common carp's immune response to various pathogens could be essential in identifying the cytokine network responsible for initiating immune responses.
- These findings have significant implications in designing effective vaccines and immunotherapies for fish diseases and understanding the fish immune system's defense mechanisms against infectious diseases.

Furthermore, the results from the immune gene expression in different carp strains (Amur wild carp, Amur Sasan, AS; Ropsha scaly carp, Rop; Prerov scaly carp, PS; and koi) infected with CEV genogroup I and genogroup IIa suggest that fish infected with both genogroups demonstrated similar expression patterns of selected immune-related genes. It is noteworthy that koi carp was the only fish strain that exhibited significant differences in the expression of most genes following infection with both CEV genogroups. Moreover, the findings suggest that koi carp's gill and gut tissues mount a systemic mucosal immune response against CEV infection, as demonstrated by the upregulation of mRNA expression for most of the analyzed immune genes. Therefore, understanding the genetic background of fish can provide valuable insights into the mechanisms underlying their immune response and susceptibility to infectious diseases.

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English summary

Susceptibility of common carp strains to the disease caused by carp edema virus

Fish diseases refer to any condition that affects the health and well-being of fish, whether in the wild or in captivity. Fish are vulnerable to a variety of diseases caused by bacteria, viruses, fungi, parasites, and other pathogens. The prevalence and severity of these diseases vary widely, depending on the species of fish, the environmental conditions, and the management practices.

Fish have a complex immune system that allows them to respond to a variety of diseases. Their immune system includes both innate and adaptive responses, which work together to protect them from infections. Innate immunity is the first line of defense and includes physical barriers such as the skin, scales, and mucus, as well as cellular and humoral responses that can quickly recognize and respond to pathogens. These responses include the activation of phagocytic cells such as macrophages and neutrophils, the release of antimicrobial peptides, and the production of reactive oxygen species. Adaptive immunity, on the other hand, is a more specific and targeted response that is mediated by T and B lymphocytes. These cells can recognize and respond to specific pathogens, and can develop immunological memory, allowing for a faster and stronger response to subsequent infections.

When a fish encounters a pathogen, its immune system is activated, and a series of responses are initiated. The exact response depends on the pathogen and the fish species, but generally involves the recruitment of immune cells to the site of infection, the activation of phagocytic cells to engulf and destroy the pathogen, and the production of cytokines to stimulate the immune response. Cytokines specially help to coordinate the immune response and ensure that the appropriate mechanisms are activated to fight the infection.

The initial investigation of the thesis provides a comprehensive review of cytokine responses in common carp exposed to CEV, CyHV-3, SVCV and *Aeromonas hydrophila* infections. It aims to synthesize existing literature on immune responses, particularly cytokine expression, and highlights the essential role of cytokines as inflammatory mediators and their mechanisms in response to both pathogenic and non-pathogenic immunostimulants in common carp. The production of these cytokines may result in various symptoms and signs of infections, which can lead to the recruitment of inflammatory cells that play an important role in clearing up different infections. Furthermore, this study provides insights into the ways by which cytokines can be used to diagnose and combat disease in common carp.

Further, the examination of the immune response of carp to CEV infection using gene expression profiling and qPCR was carried out. The expression patterns of eleven immune-related genes were monitored in four different carp strains. The expression patterns of several immune-related genes were found to be similar across all carp strains and genogroups, while some significant differences were observed in koi carp infected with both CEV genogroups. Additionally, a resemblance was observed in the expression pattern of several genes between KSD-resistant AS strain and koi. Furthermore, the observed increase in mRNA expression of several immune related genes indicates a broader mucosal immune response triggered by CEV. Overall, these results provide essential insights into the immunological response of carp to infectious diseases and may help to develop effective management strategies for preventing and controlling CEV disease in aquaculture.

Czech summary

Vnímavost plemen kapra obecného k onemocnění způsobenému kapřím edémovým virem

Nemoci ryb se týkají jakéhokoli stavu, který ovlivňuje zdraví a pohodu ryb, ať už ve volné přírodě nebo v zajetí. Ryby jsou náchylné k různým onemocněním způsobeným bakteriemi, viry, plísněmi, parazity a dalšími patogeny. Výskyt a závažnost těchto onemocnění se značně liší v závislosti na druhu ryb, podmínkách prostředí a způsobech chovu.

Ryby mají složitý imunitní systém, který jim umožňuje reagovat na různé choroby. Jejich imunitní systém zahrnuje jak vrozenou, tak získanou imunitu, které spolupracují při ochraně před infekcemi. Vrozená imunita je první linií obrany a zahrnuje fyzické bariéry, jako je kůže, šupiny a hlen, a také buněčné a humorální reakce, které dokáží rychle rozpoznat patogeny a reagovat na ně. Tyto reakce zahrnují aktivaci fagocytujících buněk, jako jsou makrofágy a neutrofily, uvolňování antimikrobiálních peptidů a produkci reaktivních forem kyslíku. Získaná imunita disponuje možností specifičtější a cílenější odpovědi, kterou zprostředkovávají T a B lymfocyty. Tyto buňky dokáží rozpoznat a reagovat na specifické patogeny a mohou si vytvořit imunologickou paměť, což umožňuje rychlejší a silnější reakci na následné infekce.

Když se ryba setká s patogenem, aktivuje se její imunitní systém a spustí se řada reakcí. Přesná reakce závisí na patogenu a druhu ryby, ale obecně zahrnuje přesun imunitních buněk do místa infekce, aktivaci fagocytárních buněk, které pohltí a zničí patogen, a produkci cytokinů, které stimulují imunitní odpověď. Cytokiny pomáhají koordinovat imunitní odpověď a zajišťují aktivaci vhodných mechanismů pro boj s infekcí.

Druhá kapitola práce poskytuje ucelený přehled cytokinových reakcí u kapra obecného vystaveného infekcím CEV, CyHV-3, SVCV a *Aeromonas hydrophila*. Jejím cílem je shrnout dosavadní literaturu o imunitních reakcích, zejména o expresi cytokinů, a zdůraznit zásadní úlohu cytokinů jako mediátorů zánětu a jejich mechanismů v reakci na patogenní i nepatogenní imunostimulátory u kapra obecného. Produkce cytokinů může vést k různým příznakům a projevům infekcí, což souvisí s aktivací zánětlivých buněk, které hrají důležitou roli při odstraňování různých infekcí. Kromě toho tato studie přináší poznatky o způsobech, jakými lze cytokiny využít k diagnostice a potírání nemocí u kapra obecného.

Ve třetí kapitole bylo provedeno zkoumání imunitní odpovědi kapra na infekci CEV pomocí profilování genové exprese a qPCR. U čtyř různých plemen kapra byly sledovány expresní vzorce jedenácti genů souvisejících s imunitou. Bylo zjištěno, že expresní vzorce několika genů souvisejících s imunitou jsou podobné u všech plemen "nebarevných" kaprů, zatímco u kaprů koi infikovaných oběma genovými skupinami CEV byly pozorovány některé významné rozdíly. Kromě toho byla pozorována podobnost ve vzorci exprese několika genů mezi Amurským sazanem, odolným vůči spavé nemoci koi kaprů, a koi kaprem. Kromě toho pozorované zvýšení exprese mRNA několika genů souvisejících s imunitou naznačuje širší slizniční imunitní odpověď vyvolanou CEV. Celkově tyto výsledky poskytují zásadní poznatky o imunologické reakci kaprů na infekční onemocnění a mohou pomoci při vývoji účinných strategií prevence a kontroly onemocnění CEV v akvakultuře.

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List of publications

Peer-reviewed journals with IF

Papežíková, I., Piačková, V., Dyková, I., **Baloch, A. A.**, Kocour Kroupová, H., Zusková, E., Pojezdal, Ľ., Minářová, H., Syrová, E., Banďouchová, H., Hyršl, P., Matějíčková, K., Pikula, J., Palíková, M., 2023. Clinical and laboratory parameters of carp edema virus disease : A case report. Viruses-Basel 15, 1044. (IF 2022 = 4.7; AIS 2022 = 1.094)

Baloch, A. A., Steinhagen, D, Gela, D, Kocour, M, Piačková, V, Adamek, M., 2023. Immune responses in carp strains with different susceptibility to carp edema virus disease. PeerJ. 10.7717/peerj.15614 (IF 2022 = 2.7; AIS 2022 = 0.709)

Baloch, A. A., Abdelsalam, E. E. E., Piačková, V., 2022. Cytokines studied in carp (*Cyprinus carpio* L.) in response to important diseases. Fishes 7(1), 3.(IF 2021 = 3.170; AIS 2012 **=** 0.503)

Manuscripts

Khoso, P., Memon, A., **Baloch, A. A.**, Mangi, R., Khoso, Z., Effect of minerals and vitamins supplementation on performance and hematological values in broiler. (accepted, Journal of Northeast Agricultural University, English Edition)

Peer-reviewed journals without IF

Bughio, E., Jatoi, A. S., Memon, M., Bughio, R., Khoso, P. A., Khoso, Z. A., **Baloch**, **A. A.,** 2017. Effect of age and route of administration on the efficacy of live infectious bursal disease vaccines in broiler. Sarhad Journal of Agriculture 33(2), 232–239.

Abstracts and conference proceedings

Papežíková I., Palíková M., Piačková V., **Baloch A. A**., Pojezdal L., Syrová E., Minářová H., 2020. Hematologické, biochemické a imunitní parametry u ryb **s** edémovou nemocí kaprů. Sborník XVII. Rybářské a ichtyologické konference, 4.–5. 11.2020, České Budějovice, s. 44.

Baloch, A. A., Piačková, V., 2019. Susceptibility of common carp strains to the disease caused by carp edema virus. Zebra Fish Immunology/Vaccination conference. Wageningen. April 24. May 2, 2019. (Poster presentation)

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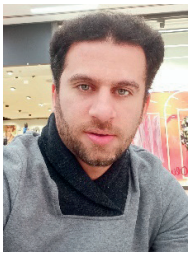

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