RESEARCH **7**

2019-163

WILL CARP VIRUS BIOCONTROL BE EFFECTIVE?

NATIONAL CARP CONTROL PLAN

Understanding the genetics and genomics of carp strains and susceptibility to CyHV-3

This suite of documents contains those listed below.

NCCP TECHNICAL PAPERS

1. Carp biocontrol background
2. Epidemiology and release strategies
3. Carp biocontrol and water quality
4. Carp virus species specificity
5. Potential socio-economic impacts of carp biocontrol
6. NCCP implementation
7. NCCP engagement report
8. NCCP Murray and Murrumbidgee case study
9. NCCP Lachlan case study

NCCP RESEARCH (peer reviewed)

Will carp virus biocontrol be effective?

1. 2016-153: Preparing for Cyprinid herpesvirus 3: A carp biomass estimate for eastern Australia
2. 2018-120: Population dynamics and carp biomass estimates for Australia
3. 2017-148: Exploring genetic biocontrol options that could work synergistically with the carp virus
4. 2016-170: Development of hydrological, ecological and epidemiological modelling
5. 2017-135: Essential studies on Cyprinid herpesvirus 3 (CyHV-3) prior to release of the virus in Australian waters
6. 2020-104: Evaluating the role of direct fish-to-fish contact on horizontal transmission of koi herpesvirus
7. 2019-163 Understanding the genetics and genomics of carp strains and susceptibility to CyHV-3
8. 2017-094: Review of carp control via commercial exploitation

What are the carp virus biocontrol risks and how can they be managed?

9. 2017-055 and 2017-056: Water-quality risk assessment of carp biocontrol for Australian waterways
10. 2016-183: Cyprinid herpesvirus 3 and its relevance to humans
11. 2017-127: Defining best practice for viral susceptibility testing of non-target species to Cyprinid herpesvirus 3
12. 2019-176: Determination of the susceptibility of Silver Perch, Murray Cod and Rainbow Trout to infection with CyHV-3
13. 2016-152 and 2018-189: The socio-economic impact assessment and stakeholder engagement
    Appendix 1: Getting the National Carp Control Plan right: Ensuring the plan addresses community and stakeholder needs, interests and concerns
    Appendix 2: Findings of community attitude surveys
    Appendix 3: Socio-economic impact assessment — commercial carp fishers
    Appendix 4: Socio-economic impact assessment — tourism sector
    Appendix 5: Stakeholder interviews
    Appendix 6: Socio-economic impact assessment — native fish breeders and growers
    Appendix 7: Socio-economic impact assessment — recreational fishing sector
    Appendix 8: Socio-economic impact assessment — koi hobbyists and businesses
    Appendix 9: Engaging with the NCCP: Summary of a stakeholder workshop
14. 2017-237: Risks, costs and water industry response
15. 2017-054: Social, economic and ecological risk assessment for use of Cyprinid herpesvirus 3 (CyHV-3) for carp biocontrol in Australia
    Volume 1: Review of the literature, outbreak scenarios, exposure pathways and case studies
    Volume 2: Assessment of risks to Matters of National Environmental Significance
    Volume 3: Assessment of social risks
16. 2016-158: Development of strategies to optimise release and clean-up strategies
17. 2016-180: Assessment of options for utilisation of virus-infected carp
18. 2017-104: The likely medium- to long-term ecological outcomes of major carp population reductions
19. 2016-132: Expected benefits and costs associated with carp control in the Murray-Darling Basin

NCCP PLANNING INVESTIGATIONS

1. 2018-112: Carp questionnaire survey and community mapping tool
2. 2018-190: Biosecurity strategy for the koi (*Cyprinus carpio*) industry
3. 2017-222: Engineering options for the NCCP
4. NCCP Lachlan case study (in house) (refer to Technical Paper 9)
5. 2018-209: Various NCCP operations case studies for the Murray and Murrumbidgee river systems (refer to Technical Paper 8)

FRDC
FISHERIES RESEARCH & DEVELOPMENT CORPORATION

DRAFT FINAL REPORT

Understanding the genetics and genomics of carp strains and susceptibility to CyHV-3

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Contents

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Abbreviations

CC: Common carp (*Cyprinus carpio*)

CKMR: Close kin mark recapture

CyHV-3: Cyprinid herpesvirus 3

Cyt-Cyt: Cytokine-cytokine (interaction)

DEG: Differentially expressed genes

GBS: Genotyping by sequencing

GO: Gene Ontology

KEGG: Kyoto Encyclopedia of Genes and Genomes

KHV: Koi herpesvirus

MU: Management units

n/a: not applicable

NCBI: National Center for Biotechnology Information

NCCP: National Carp Control Plan

NGS: next generation sequencing

NSW: New South Wales

PCA: Principal components analysis

QTL: Quantitative trait loci

RNA-Seq: RNA Sequencing

SNP: single nucleotide polymorphism

ZF: Zebrafish (*Danio rerio*)

Executive Summary

- We report on a pilot study investigating the use of immunogenetic profiling to inform the risk that common carp in south-eastern Australia might develop rapid resistance to CyHV-3 if this were to be released as a biocontrol agent.

- The justification of this study are assertions in the scientific literature that such development of resistance will occur rapidly following the virus release, and the resulting rebound in the common carp population will thus negate any benefits arising from the use of CyHV-3.

- DNA samples were collected from strains of common carp in rivers and waterways of south-eastern Australia, as well as from a population of feral koi carp in the Sydney area. In addition, feral goldfish and goldfish hybrid samples were sourced from known populations. The Australian sampling was supplemented with aquaculture strains of varying resistance from the Czech Republic and Israel.

- Next gen sequencing (NGS) was undertaken from the 41 Australian, Czech and Israeli samples, and high-performance computing was applied to assemble the reads and extract a file of single-nucleotide polymorphisms (SNPs) for 29 immune genes, which a recent study had identified as being associated with resistance to CyHV-3.

- Applying the standard method of Principal Components Analysis (PCA) to the whole SNP immunogenetic dataset, we were able to detect three major clusters which corresponded to resistant goldfish, resistant goldfish x carp hybrids and variably susceptible common and koi carp.

- Reapplying the analysis to just the common and koi carp collected from Australia and Israel showed a distinct clustering, with one grouping corresponding to strains derived from European aquaculture – which include the invasive Australia carp – and a second grouping of the highly resistant Amur Sassan strain. This was interpreted as a lack of evidence that the Australian strains possessed the alleles which would enable the rapid development of innate immune mediated resistance.

- Whilst the majority of the Czech samples supported this division into resistant and susceptible immune-genotypes, there were however some noticeable inconsistencies. In particular, one presumed resistant Amur scaly sample clustered very closely to a susceptible Rhine carp. An explanation for this and other inconsistencies is not apparent but indicates the need for a follow up confirmatory studies, including comparing the immunogenetics of surviving and non-surviving invasive Australian carp experimentally challenged with CyHV-3.

- The study reported here lays the foundation for a low-cost SNP-based method to monitor the development of resistance to CyHV-3 if a release is eventually decided to proceed. Furthermore, this monitoring could be integrated with a recently developed SNP based method – close kin mark recapture - to estimate carp population size, and thus deliver an integrated system for the ongoing assessment of the field effectiveness of the virus as a sustainable biocontrol agent.

Keywords

Biocontrol, *Carassius auratus*, *Cyprinid herpesvirus 3*, *Cyprinus carpio*, *Danio rerio*, Immunogenetics

Introduction

Common carp (*Cyprinus carpio*) – hereafter referred to as carp - are an invasive species of the rivers and waterways of south-eastern Australia, implicated in the serious decline of many native fish species (Koehn 2004). Over the past 50 years, a variety of control options have been explored, all of which to date have proved either ineffective or cost prohibitive. Most recently, cyprinid herpesvirus-3 (CyHV-3) has been proposed as a biocontrol agent based on it causing a relatively high mortality and as well as being specific to carp (McColl et al. 2017).

To enable planning for an optimal release of the virus to achieve maximum benefit we undertook integrated ecological-epidemiological modelling (Durr et al. 2019). This modelling indicated that whilst CyHV-3 could achieve sustained reductions in the population, we stressed that this result assumed that the virus would over a 5–10 year period continue to cause a mortality in infected carp of 60-80%, i.e. no immediate resistance to infection or mortality would arise.

However, recent scientific papers have questioned whether this assumption is realistic, warning that the development of immunological and/or innate immunity might be inevitable (Marshall et al. 2018; Becker et al. 2019; Kopf et al. 2019). Accordingly, this raises questions as to the long-term benefit of releasing the virus. To assess the risk that rapid resistance to CyHV-3 might develop, we undertook an extension to the integrated modelling with the objectives of defining what exactly is “resistance” in the context of viral biocontrol, and to elucidate the mechanisms (pathways) by which it might develop. This was achieved through both an extensive literature review (Samsing et al. 2021) as well as forward-time population genetics simulation modelling (Durr et al. 2020).

The main conclusion of both the literature review and the simulation modelling was that the most plausible means by which carp might have a successively reducing mortality rate following a release of CyHV-3 would be through selection for resistance conferring forms of the genes (“alleles”) already present in the genomes of Australian carp population. By contrast, the other two identified pathways, viz. (1) *de novo* (spontaneous) mutations leading to alleles conferring resistance and (2) hybridisation of carp with a closely related cyprinid species which are resistant to CyHV-3 like goldfish would require, respectively, a very long timescale to develop or would result in a hybrid population with reduced ecological fitness.

Redefining an assessment of the risk that common carp will rapidly develop resistance to CyHV-3 to be one of determining the extent to which resistant alleles are present in the Australian carp population simplifies the problem but determining this in practice presents its own challenges. Carp in Australia have a complex history of introduction and although three major strains are identifiable there now exists geographically definable sub-populations whose genetic composition differs in the proportion of these strains (Haynes et al. 2009; Haynes et al. 2010; Haynes et al. 2012). Thus, to rigorously demonstrate the presence of resistant sub-populations through experimental challenge trials would require that such trials be repeated over more than 20 sub-populations. Moreover, the results of the challenge trial undertaken on carp as part of the non-target species testing showed considerable variability in the percentage mortality depending on the challenge route and the geographical source of the fish (McColl et al. 2017). Therefore, to provide definitive proof of the existence or conversely the lack of fully or partially resistant carp sub-populations would require a very large number of challenge trials allowing for adequate replication as well as the need to varying such factors as the infection route, the source, age and the genetic structure with respect to resistance to CyHV-3.

Of these variables, it is the genetic structure with respect to CyHV-3 resistance for which there is most uncertainty. The studies by Haynes et al. (2009) and Haynes et al. (2010) assigned carp sub-

populations by measuring repeat-length variability in 14 microsatellite loci, which was assessed using fragment size of DNA extracted using conventional PCR. Since then, there has been substantive progress in determining genotypes due to the development of relatively low-cost, high-throughput ("next-gen") sequencing. This has enabled population-level genotyping based on single nucleotide polymorphisms (SNPs) to become the standard method of sub-population delineation (Seeb et al. 2011). However, in fisheries, SNP-based genotyping is usually applied to define sub-populations based on variation of loci throughout the whole genome (i.e. genotyping by sequencing) and less frequently on specific genes except where these have been robustly associated with disease resistance (Samsing et al. 2021).

Fortunately, the defining of the genetic basis of carp resistance to CyHV-3 has made considerable progress in the past 10-15 years (Samsing et al. 2021). The motivation for this research has been the ongoing threat the virus poses to aquaculture and the potential of introgressing CyHV-3 resistance alleles into improved, domesticated carp lineages as a means of control. The basis of much of the latter work follows from the discovery that a commercial breed ("Sassan") derived from a wild strain of carp found in the Amur River region of northeast Asia is relatively resistant to infection to CyHV-3 (Shapira et al. 2005). Initially it was even suggested that single genes or gene groups might act as markers for CyHV-3 resistance (Rakus et al. 2009; Kongchum et al. 2011), but it is now recognised to be a complex, polygenic quantitative trait (Samsing et al. 2021).

Given the progress made in the past 10 years in immunogenetics and next-gen sequencing, there is now the possibility to characterise the invasive carp populations in Australia with respect to the presence of alleles for CyHV-3 resistance. However, there are many technical challenges to overcome before this potential might become a reality, including the fact that that the carp genome, despite being published in 2014, is still at a semi-draft stage with its assemblies discontinuous and comprised of many scaffolds (Xu et al. 2014). Furthermore, the annotation of the genome is incomplete, relying to a large extent on identifying comparable genes in the zebrafish, *Danio rerio,* which is justified by this also being a cyprinid, but ignores the tetraploidy of common carp (Li et al. 2015). Finally, the actual genetic architecture of resistance of carp to CyHV-3 is still under active investigation by various research teams and will require several more years of research before it is fully understood (Palaiokostas et al. 2019; Tadmor-Levi et al. 2019b; Jia et al. 2021).

Objectives

The overall objective of this study was to explore the potential of a next-gen, immunogenetic approach to provide insight into the potential of carp to develop rapid resistance to CyHV-3 if it were to be released as a bio-control agent to suppress populations of this invasive species in south-eastern Australia.

The more specific objectives, as agreed to in the project contract, were to:

1. Determine the likely genes responsible for resistance of carp to CyHV-3.

2. Undertake whole genome sequencing of strains of carp and hybrids present in Australia and overseas.

3. Undertake bioinformatics analyses to assess the likelihood of resistance conferring forms of genes ("alleles") being present in Australian strains.

MethodsSelection of candidate genes for resistance to CyHV-3

To compile a list of candidate genes which might collectively define Australian strains of carp as being susceptible or relatively resistant to CyHV-3, we used quantitative trait loci (QTL) mapping exercise described in Tadmor-Levi et al. (2019b). This study used NGS and SNP markers to associate survival following CyHV-3 infection (the phenotypic trait) with varying introgression with the relatively resistant Amur-Sassan strain of carp. This successfully identified four QTL regions of the genome associated with relative resistance. Of these, the two principal ones (QTL1 and QTL2) mapped to common carp (CC) linkage map 30 and 46, and by comparison to the better characterised zebrafish (ZF) genome, a list of over 1700 genes were identified in these QTLs. Based on the ZF gene IDs and annotations, gene ontology (GO) terms analysis assigned carp genes to GO term categories, and this enabled 35 carp immunity-related genes to be identified in the *Ensembl* ZF genome database (Release 104), although two of these have since been listed as "retired" by *Ensembl* thus reducing the final list to 33 ZF genes.

Tadmor-Levi et al. (2019b) did not specifically identify the homologous CC genes for the 33 ZF immune genes, and to resolve this we used the "Orthologues" function of *Ensembl* (Herrero et al. 2016). This enabled 30 CC orthologous / paralogous genes to be identified which the *Ensembl* pipeline identified as being of "sufficient confidence". As two ZF IL10 paralogues were identified, our final list of putative CC CyHV-3 resistance genes was reduced to 29, for which we extracted the SNPs from each of the sequenced genomes (see below).

As a final step to determine the functional role of the candidate genes, we used the *Ensembl* gene name to identify the NCBI gene identifier, and where this was found, we used it to query the *KEGG GENES* database (Kanehisa et al. 2017).

Collection of DNA from feral common carp and goldfish-carp hybrids in Australia

The basis for an informed sampling of feral common carp and its hybrids was the population genetics studies reported by Haynes et al. (2009) and Haynes et al. (2010), in which over 1,000 carp samples were collected from catchments in south-eastern Australia and genotyped. This identified 4 parental introductions - Prospect, Yanco, Boolarra and feral Koi - with the former three being of European aquaculture provenance and Koi from Asia. Furthermore, whilst there has been extensive mixing of the lineages, geographical patterns were found representing the routes of introduction, with Yanco and Prospect being via the Sydney region and Boolarra via the Gippsland region east of Melbourne. Haynes et al. (2012) also confirmed hybridisation between carp and goldfish, although this occurred mainly in a restricted area at the headwaters of the Macquarie River. These hybrids are of particular interest, as this has been suggested as a possible pathway for the development of rapid resistance to CyHV-3 (Mintram et al. 2021).

Based on this information, we aimed to purposively sample carp in catchments where we would expect to find carp with a predominance of the four lineages, as well as the specific rivers and lakes where hybrids have been reported. This sampling was mostly undertaken in collaboration with commercial fishermen with extensive experience of carp.

For all sampling, a sliver of a fleshy part of the dorsal fin-clip was requested with preservation in 70% alcohol.

Collection of DNA from overseas aquaculture strains of carp

As noted above, the possibility of breeding CyHV-3 tolerant brood-stock by introgressing alleles from resistant strains has been an objective for sustainable and cost-effective control of the disease in commercial aquaculture. In Europe an important institute for research in this area has been the University of South Bohemia in České Budějovice on account of their extensive collection of common carp strains, many of which have been genetically characterised (Hulak et al. 2010).

Following the advice of Prof. Martin Flajšhan at this University, three strains were selected for sequencing, representing varying presumed resistance to CyHV-3:

1. Amur scaly carp representing carp considered to be most resistant to CyHV-3.
2. Amur mirror carp with presumed intermediate resistance.
3. Rhine wild carp with presumed least resistance.

Following sequencing and analysis of these Czech strains, we found an unexpected lack of clustering of strains with respect to CyHV-3 resistance (see Results below). Whilst not discounting that this was a real phenomenon, we hypothesised that this might be due to these strains having inherent variability with respect to CyHV-3 innate resistance, which has support from the literature (Odegard et al. 2010; Tadmor-Levi et al. 2017; Palaiokostas et al. 2019). Thus, to accurately characterise the latter we might need to select specific families within lineages.

Accordingly, we undertook a second round of overseas sampling, developing a collaboration with Dr Lior David at the Hebrew University of Jerusalem in Israel. Of note, Dr David was the lead scientist for the QTL study undertaken by Tadmor-Levi et al. (2019b) which we used to define the CyHV-3 resistant genes.

Based on the advice of Dr Lior David, families from the following lineages were sampled:

1. Amur Sassan – high resistance to CyHV-3.
2. Dor 70 – intermediate resistance.
3. Koi carp – low resistance.

DNA extraction and next-gen sequencing of carp DNA

For the samples collected from the fin-clips of carp in Australia, DNA was extracted and assessed using standard methods. DNA was extracted from fin samples stored in ethanol using the Qiagen's DNeasy Blood and Tissue Kits catalog number 69504 following the manufacturer's protocol. The concentration of DNA was measured using a NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific Inc., USA) at ACDP and QuantiFluor® dsDNA System (Promega) at AGRF following the manufacturer's protocols. The quality of DNA was assessed using 1% E-Gel Precast Agarose Electrophoresis System (Invitrogen) following the manufacturer's protocol.

Based on quality problems being encountered for the extracted DNA from the fin clips from the Australian fish, DNA extraction was undertaken from the blood samples by the supplying institutes for the Czech and the Israeli carp strains.

Three rounds of whole genome, next-gen sequencing were undertaken, using two sequencing companies:

- AGRF Melbourne – all DNA from the SE Australia sampling and the Israeli sampling.

- Macrogen South Korea – the extracted DNA from the Czech samples

All sequencing runs were undertaken using the Illumina NovaSeq 6000 platform (https://support.illumina.com/sequencing/sequencing_instruments/novaseq-6000/documentation.html).

NGS quality assessment and resistant gene and allele variant extraction

The raw reads from the Illumina sequencing were first compared to the 29 candidate immune genes (see Results below), and matching reads were extracted for downstream analysis using *bbmap* v.38.37 (Bushnell 2014) with the default parameters.

The extracted reads were then cleaned using *Trimmomatic* v.0.38 (Bolger et al. 2014) by removing Illumina adapters, trimming when the quality dropped below 20, and discarding remaining reads shorter than 50 bp.

High-quality reads were then aligned to the 29 candidate immune genes using *STAR* v.2.7.0 (Dobin et al. 2013) with the default parameters.

In order to call SNPs from the resulting bam files, read group library, platform, platform unit and sample name were added to the files with *Picard* v.2.9.2 (http://broadinstitute.github.io/picard).

SNPs were then called for each sample using *bcftools* v.1.9.0 (Li et al. 2009) with the *mpileup*, *call*, then *filter* sub-commands. Final SNPs were filtered if they had a quality score lower than 20 or coverage less than 100 and a combined VCF file outputted.

In the initial stage of implementing the above pipeline, we undertook a formal analysis of the quality of the NGS reads and the accuracy of the pipeline to correctly identify SNPs. For this assessment we used the read data arising from both the first sequencing run done at AGRF ("AUS1") and the second run undertaken at Macrogen ("AUS2) and compared the NGS gene depth of coverage to that of four unassembled genomes deposited in the NCBI's Short Read Archive (SRA) as part of the analyses (Xu et al. 2014) that reported the genome of common carp (Bioproject PRJNA202478). As a follow-on study, we then assessed the pipeline for its accuracy and replicability to detect SNPs, comparing the read depth, coverage and detection of SNPs for the toll-like receptor 1 (tlr1) gene for three replicates of fish presumed to be the same genotype collected from NSW, the Czech Republic and Koi, the latter deposited in the SRA by Xu et al. (2014).

Assessment of population structure with respect to resistant genes

To assess the population structure of the 41 sequenced cyprinid fish based on the alleles (SNPs) of the 29 purported resistance genes, we used Principal Components Analysis (PCA). The input of this was the VCF file (see above), from which we used *PLINK* v.1.9.0 (Chang et al. 2015) to extract the eigenvectors (for 2-dimensional display) and the eigenvalues (for the relative importance of the principal components). The graphing of the first two principal components (PCs) – i.e. PC1 vs PC2 was undertaken using *ggplot2* with R (Wickham 2016).

Results

Candidate genes for resistance to CyHV-3

Using the data of the 35 immune zebrafish gene *Ensembl* IDs which Tadmor-Levi et al. (2019b) deduced to be related to CyHV-3 resistance in carp, we were able to find 29 CC orthologous genes in the *Ensembl* genome database version 102 (Table 1). As expected, considering both species are cyprinids, there was a high cDNA percentage identity between these genes for the two species, averaging 73.1%.

Regarding the functional role of these genes, only nine had a KEGG pathway term assigned, and for these the overwhelming majority (7/9) it was “cytokine-cytokine receptor interaction”.

Carp, goldfish and carp x goldfish strains sampled for DNA

In total, tissue from 36 fish were sampled from southeast Australia, of which 28 were identified by the fishers to be carp, 6 to be goldfish and 2 as carp x goldfish hybrids (Table 2). Of the carp, all four of the phenotypes identified by Haynes et al. (2009), i.e. Boolarra, Yanco, Koi and Prospect were sampled. However, for the sampling for the Prospect phenotype, the supplying fisher considered that they possibly were Prospect x Boolarra hybrids. Of the 36 sampled fish, 22 had their tissue sent for sequencing.

From the University of South Bohemia, we obtained 3 representatives from each phenotype, and from the Hebrew University of Jerusalem we obtained 4 sample each from the two carp strains and 2 samples from the Koi strains, i.e. 19 overseas samples in total. All these samples were sent for sequencing.

Validation of SNP extraction bioinformatic pipeline and assessment of read depth and SNP calling

The formal comparison of the read depth of coverage for the first two batches of sequencing – undertaken at AGRF (Australia) and Macrogen (Korea) – with reference sequences arising from the common carp complete genome project (Xu et al. 2014) showed that the average depth obtained in our runs exceeded those of the latter by a factor consistently greater than 2 for all but one gene (Figure 1). The follow-on analysis of the tlr1 gene confirmed this high depth and also showed good coverage across the length of the gene (Figure 2). The replicability of the detected SNPs was also high between the three sequenced carp of the same phenotype, i.e. Amur Mirror carp (collected from the Czech Republic) and Yanco (collected from NSW).

Population structure of immune genes of common carp and carp x goldfish hybrids

The PCA plots of the SNPs from the immune genes of Table 1 for the total 41 sequenced genomes of carp, goldfish and their hybrids collected from southeast Australia, the Czech Republic and Israel showed a clear population structuring (Figure 3a). Specifically, the goldfish and the goldfish hybrids formed separate clusters from the common carp for all but one Boolarra strain (Figure 3b). This was almost certainly misidentified, as “cryptic hybridisation” between carp and goldfish is a well-recognised phenomena in south-eastern Australia (Haynes et al. 2012). Of note, the sampling location of this presumed hybrid was in a reservoir in the Latrobe River catchment where Hume et al.

(1983) had identified hybrids in the 1980s. Both these clusters are presumed to have a high resistance to CyHV-3 (Figure 3c) based on the results of infection trials, albeit conducted outside of Australia (Hedrick et al. 2006; Yuasa et al. 2013).

Although common and koi carp formed a distinct cluster as compared to goldfish and the goldfish hybrids, the structuring was more complex as reflects the diversity of strains sampled (Figure 3b). The most clearly differentiated were the two cultured Koi carp from Israel, which however, did not group with the feral koi collected from Sydney which were contained within the common carp cluster (Figure 4b). This is readily explained by the feral Australian koi having undergone extensive hybridisation with common carp strains over an extended period, albeit retaining the distinctive skin colouration which are characteristic of this genotype.

Of the common carp strains, those collected from both NSW and VIC formed a very tight immunogenetic cluster (Figure 4a). This was somewhat surprising considering the different provenance of carp in the two states, but possibly reflects the introgression of the Boolarra strain into the two traditional NSW strains, i.e. Prospect and Yanco (Figure 4b).

Of the international reference strains, those from Israel showed a clear pattern, with the highly resistant Amur Sassan forming a distinct cluster from the more susceptible Dor-70, the later clustering closely to the Australian invasive carp strains (Figure 4b). This closeness of the immunogenetic profiles between the Australian feral carp and Dor-70 is as expected, as although the later has a complex origin, it is mainly derived from Western European strains (Wohlfarth et al. 1980).

As the sampling from the Israeli strains was from families that have been characterised with respect to CyHV-3 challenge, it is possible to directly infer the immunogenetic profiles with respect to resistance. Tadmor-Levi et al. (2017) reports that the challenge of two Dor-70 families resulted in a mean mortality of approximately 70%, which compares with a mean mortality (weighted on sample numbers) of 61% reported by McColl et al. (2017) from 9 challenge trials where the carp served as controls for the non-target species testing. Thus, the closeness of the immunogenetic profiles of the Australian and Dor-70 strains approximately matches their respective CyHV-3 resistance, and by extension it may be inferred that the immunogenetic separation between the susceptible Dor-70 cluster and relatively resistant Amur-Sassan cluster means that the Australian carp do not possess the alleles conferring resistance to CyHV-3.

Nevertheless, such an ordered pattern of the immunogenetic profile was not seen with the three Czech Republic samplings, with the two Amur River derived strains (i.e. Amur mirror and Amur Mirror only partially clustering with the Amur Sassan strain (from Israel). Similarly, the three Rhine carp did not from as tight a cluster, with two fish having immunogenetics distinct from the other western European derived strains (Figure 4a). An explanation for the diversity of the immunogenetics of the Czech samples is not apparent but might reflect the complex cross-breeding of wild type populations that has been undertaken to develop commercial aquaculture carp aquaculture varieties and strains (Hulak et al. 2010). However, as the sampling was undertaken from immature brood-stock carp kept in large outdoor ponds the possibility of strain misidentification, although unlikely, cannot be entirely discounted.

Table 1. Listing of 29 common carp orthologues presumed to be related to innate immunity to CyHV-3 and from which SNPs were extracted from the 31 genomes sequenced by this study. For the *KEGG* pathway assignment, "n/a" means that the CC gene was not listed in the *KEGG* database, and "not assigned" means that no pathway term was given. Where more than one *KEGG* pathway was listed, only the first one is given here.

ZF *Ensembl* ID	CC *Ensembl* Gene	Identity of	CC gene	*KEGG* Pathway

(Release 104)	Orthologue (Release 104)	CC to ZF orthologue	name	
ENSDARG00000038541	ENSCCRG00000051514	78.98%	ccr12a	Cyt-Cyt receptor interaction
ENSDARG00000052988	ENSCCRG00000020365	71.95%	xcr1b.1	Cyt-Cyt receptor interaction
ENSDARG00000058774	ENSCCRG00000020364	71.91%	none	Cyt-Cyt receptor interaction
ENSDARG00000059866	ENSCCRG00000051529	87.65%	cactin	not assigned
ENSDARG00000060322	ENSCCRG00000023675	80.45%	none	n/a
ENSDARG00000087496	ENSCCRG00000037885	60.29%	tradv30.0.6	n/a
ENSDARG00000115610	ENSCCRG00000027011	61.76%	ccl25a	Cyt-Cyt receptor interaction
ENSDARG00000090873	ENSCCRG00000026063	70.27%	none	n/a
ENSDARG00000091280	ENSCCRG00000039096	55.45%	none	n/a
ENSDARG00000092883	ENSCCRG00000023675	77.27%	none	n/a
ENSDARG00000094983	ENSCCRG00000035314	61.34%	none	n/a
ENSDARG00000101040	ENSCCRG00000016467	72.73%	ccl20a.3	Cyt-Cyt receptor interaction
ENSDARG00000004451	ENSCCRG00000012874	73.64%	tnfrsfa	Cyt-Cyt receptor interaction
ENSDARG00000032746	ENSCCRG00000024111	82.42%	tnfsf10l3	not assigned
ENSDARG00000036628	ENSCCRG00000007767	61.83%	cd74b	not assigned
ENSDARG00000044541	ENSCCRG00000000066	95.28%	ppp1r14ba	not assigned
ENSDARG00000044694	ENSCCRG00000019866	73.13%	fybb	n/a
ENSDARG00000053831	ENSCCRG00000032348	85.21%	vtnb	Focal adhesion
ENSDARG00000055955	ENSCCRG00000051798	55.32%	lcp2a	not assigned
ENSDARG00000057113	ENSCCRG00000011078	70.47%	c6	not assigned
ENSDARG00000057121	ENSCCRG00000004390	79.45%	c7b	not assigned
ENSDARG00000075161	ENSCCRG00000037329	94.03%	defbl1	n/a
ENSDARG00000077860	ENSCCRG00000029016	89.95%	ankhd1	n/a
ENSDARG00000093052	ENSCCRG00000011078	67.46%	c6	not assigned
ENSDARG00000100899	ENSCCRG00000042588	49.84%	none	Cell adhesion molecules
ENSDARG00000102367	ENSCCRG00000049510	84.14%	ndfip1l	not assigned
ENSDARG00000104045	ENSCCRG00000018092	72.40%	tlr22	not assigned
ENSDARG00000104808	ENSCCRG00000015806	66.12%	jak2a	not assigned
ENSDARG00000078147	ENSCCRG00000020358	69.02%	il10	Cyt-Cyt receptor interaction

Table 2. Sampling locations within south-east Australia for carp, goldfish and carp x goldfish hybrids.

Location	Catchment	Phenotype	Number fish sampled	Number fish sequenced
Lake Cargelligo, NSW	Lachlan	Boolarra	4	2

		Boolarra x Goldfish	2	2
		Goldfish	3	2
Edward River, NSW	Mid Murray	Boolarra	4	1
		Goldfish	3	1
Lake Burrendong, NSW	Macquarie–Castlereagh	Prospect possibly Boolarra	4	3
Laka Tala, NSW	Murrumbidgee	Yanco	4	3
Botany Wetland, Sydney NSW	Mill Stream	Koi carp (feral)	6	3
Gippsland Lakes, VIC	n/a	Boolarra	2	2
La Trobe Reservoir, Yallourn, VIC	Latrobe	Boolarra	3	2
Tarwin River, VIC	Tarwin	Boolarra	1	1
TOTAL			**36**	**22**

Figure 1. Summary of the exercise undertaken to validate the gene and SNP extraction pipeline from the NGS reads showing the average coverage of reads over 20 immuno-genes. The group AUS1 refers to the first batch of Australian carp sequenced at the AGRF (n=14), and AUS2 refers to a batch sequenced at Macrogen (n=9). The other four carp refer to genomes downloaded from NCBI's SRA BioProject PRJNA202478 (Xu et al. 2014). Note that the immuno-genes displayed do not correspond to those in Table 1 as these were chosen on the basis of a literature review and before the decision to use those suggested by the genetic study of Tadmor-Levi et al. (2019b).

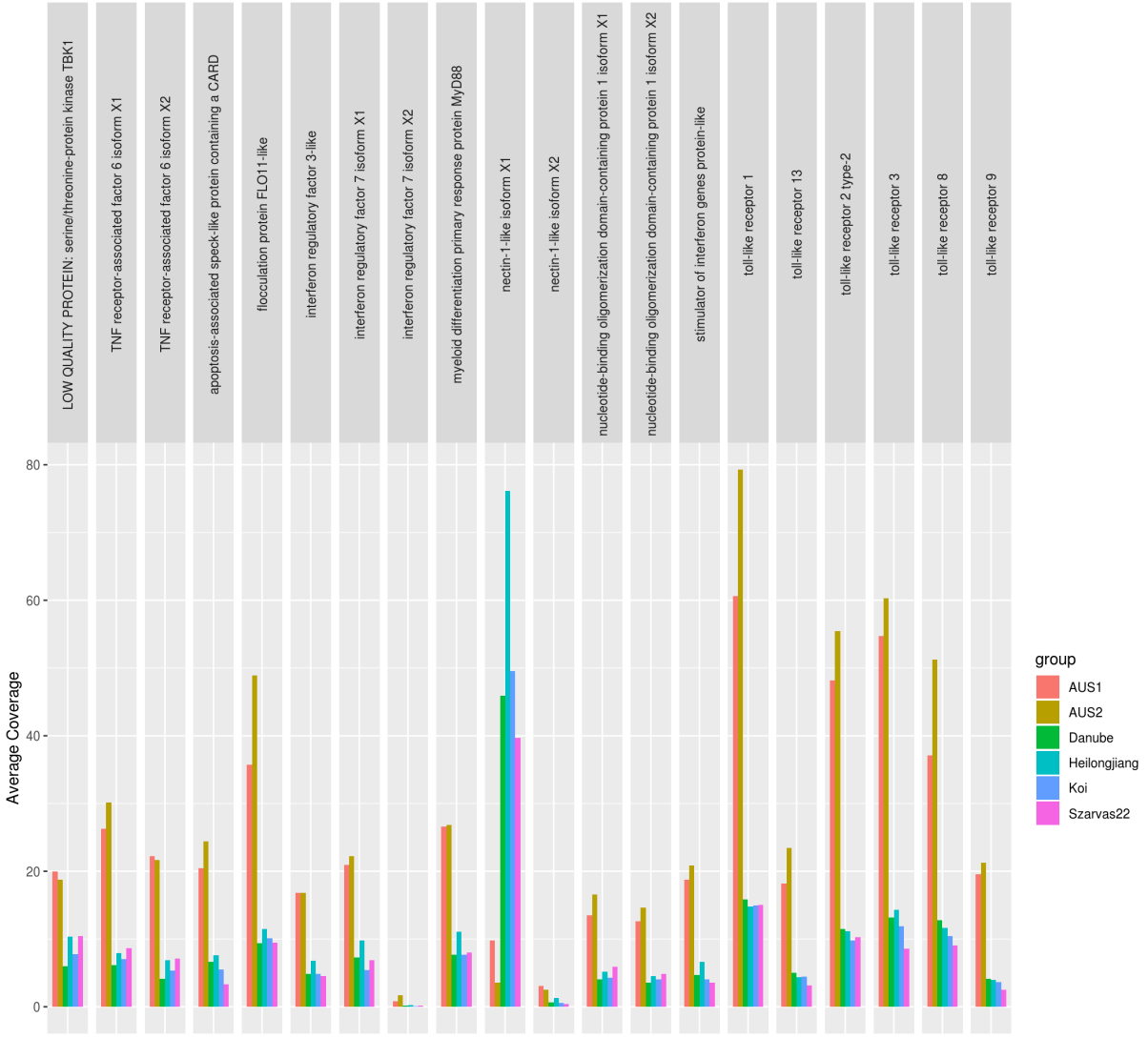


Figure 2. SNPs called over the tlr1 gene comparing 3 Amur mirror carp (AM1-3: “AUS2”) from the Czech Republic sequenced by Macrogen, 3 invasive common carp (Y1-3: “AUS1”) from NSW sequenced by AGRF and 3 Koi carp (K1-3 “Koi”) sequenced for the carp genome project (BioProject PRJNA202478). The coloured area of the graph indicates the read coverage while the vertical lines indicate SNPs. Allele symbols indicate the type of SNP as per standard vcf files.

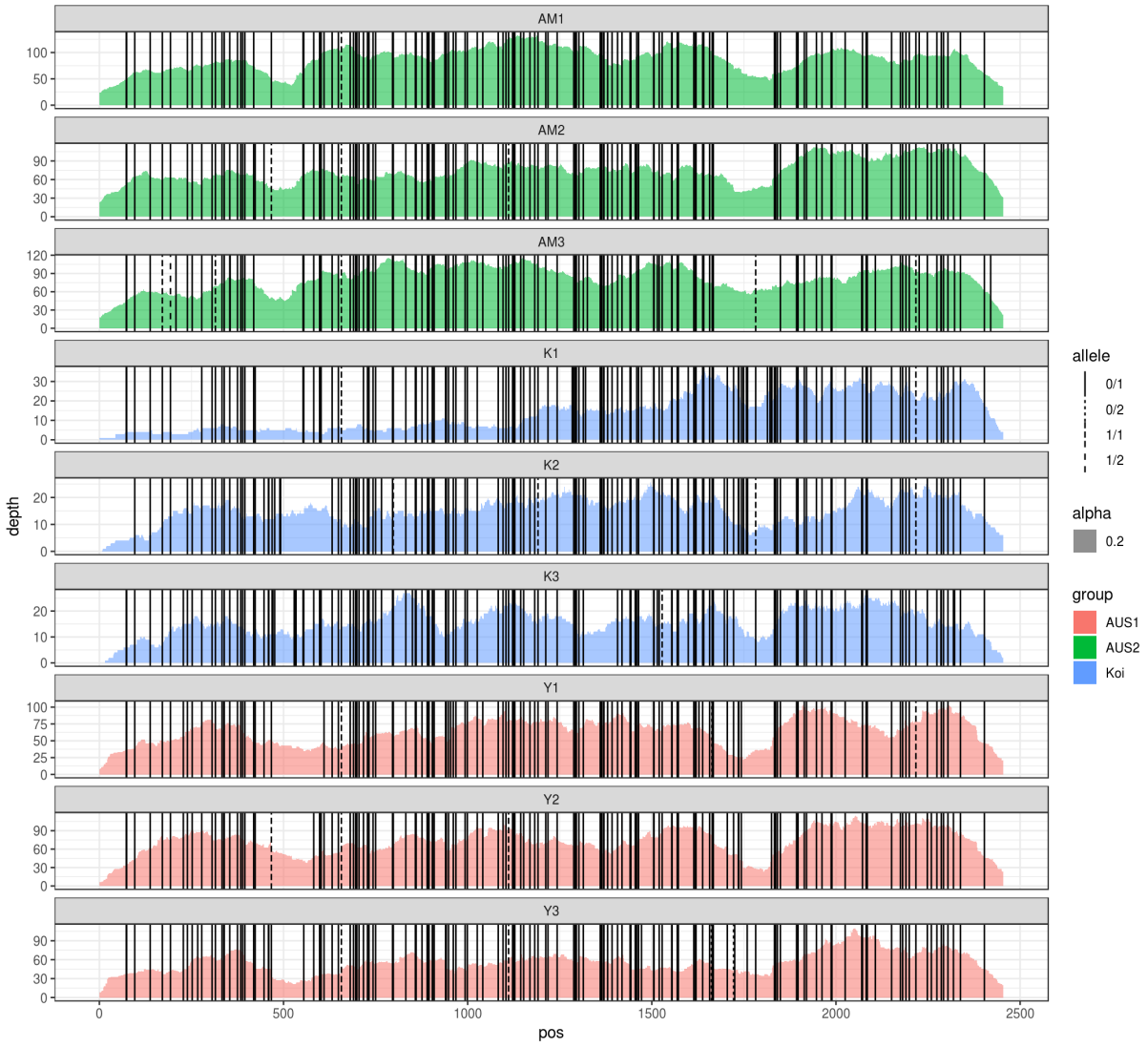


Figure 3. Principal Components Analysis plots of the 29 immune genes SNPs from the 41 sequenced common carp, goldfish and hybrids collected from south-eastern Australia (n = 22), the Czech Republic (n = 9) and Israel (n = 10). All three plots show the eigenvectors for PC1 and PC2 but are coloured according to different attributes of the sampled fish, i.e. (a) provenance; (b) phenotype; and (c) presumed resistance to CyHV-3. The percentage of the total variance explained by PC1 and PC2 are 56% and 7% respectively.

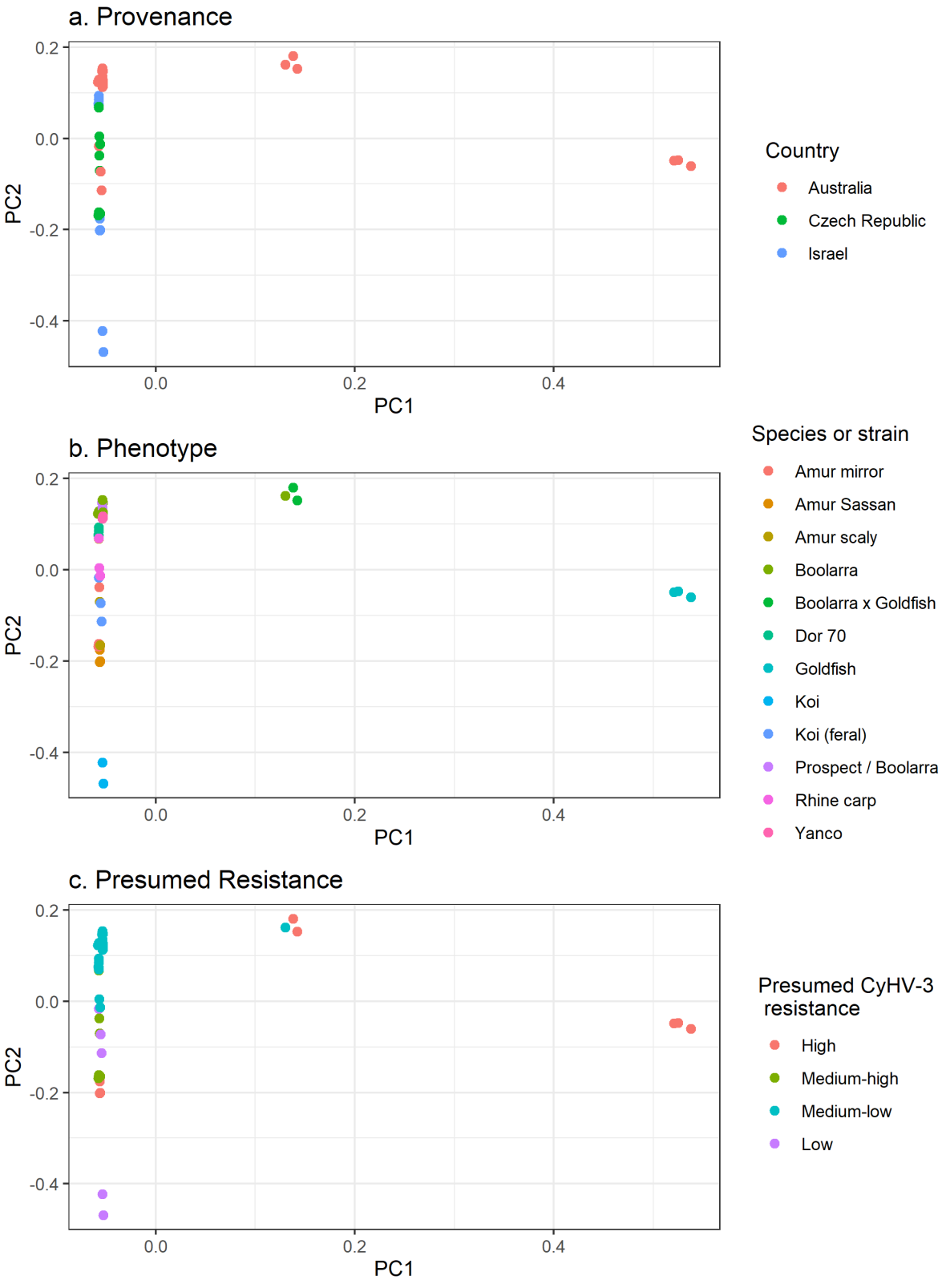
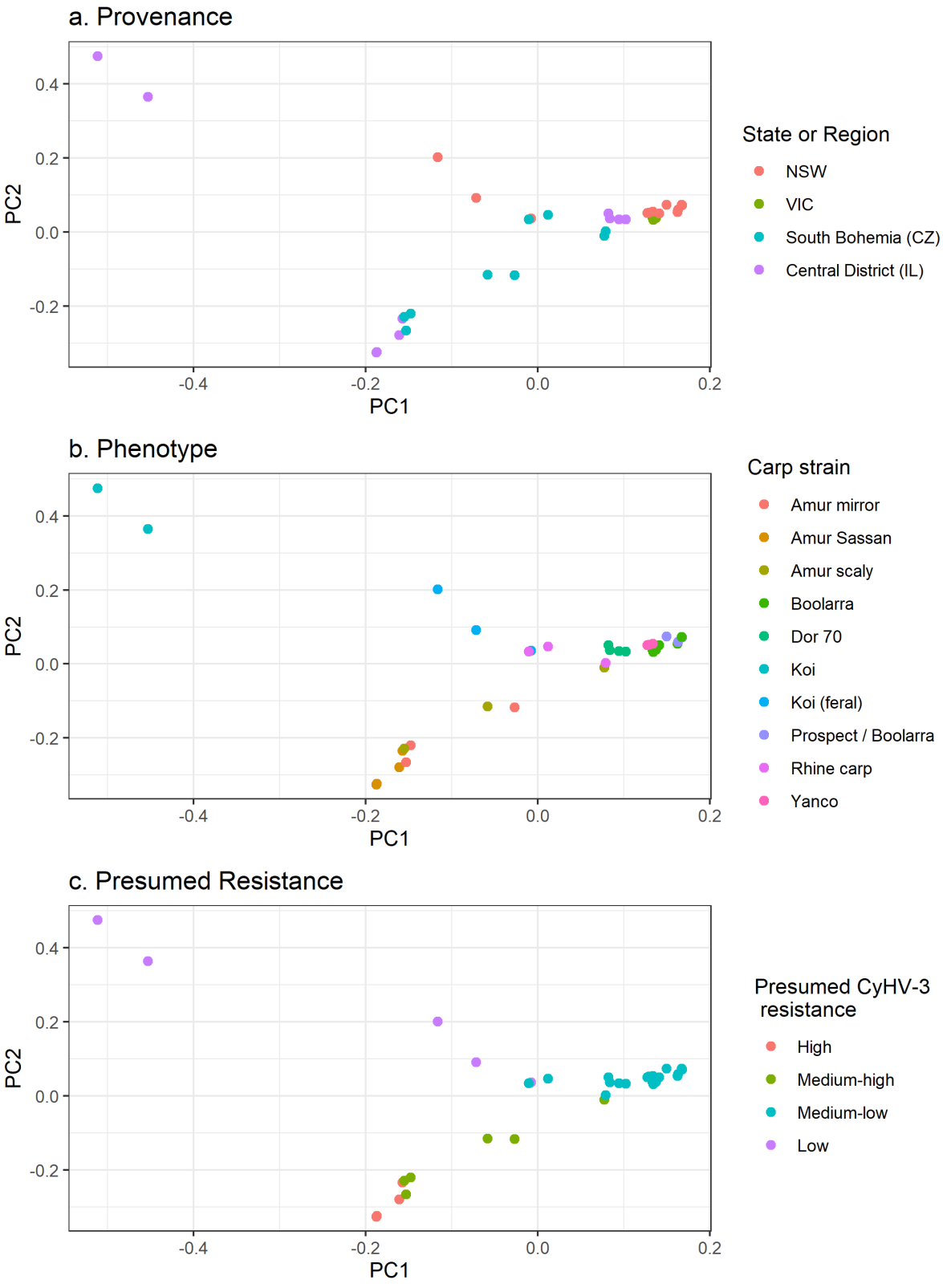


Figure 4. Principal Components Analysis plots of the 29 immune genes SNPs from the 35 sequenced common carp collected from south-eastern Australia (n=16), the Czech Republic (n = 9) and Israel (n=10). All three plots show the eigenvectors for PC1 and PC2 but are coloured according to different attributes of the sampled fish, i.e. (a) provenance; (b) phenotype; and (c) presumed resistance to CyHV-3. The percentage of the total variance explained by PC1 and PC2 are 18% and 14% respectively.



Discussion

The underlying rationale for the study described in this report is the importance of providing scientific evidence to inform an assessment of whether rapid resistance to CyHV-3 by invasive common carp will develop if the virus were to be released. With the overall conclusion from the ecological and epidemiological studies commissioned by the National Carp Control Plan (NCCP) that a release of the virus is technically feasible and that the environmental risks are minimal or at least manageable, the issue of resistance – or more correctly the rate at which it might develop – has become of paramount concern (Boutier et al. 2019; McColl and Sunarto 2020).

The approach we adopted to explore this rate of resistance development has been incremental. First, we undertook a detailed inter-disciplinary literature review which clearly demonstrated the complexity of host resistance and the large number of issues that needed to consider as well as the considerable knowledge gaps (Samsing et al. 2021). However, a key insight from this review is that there are only three pathways by which resistance can develop, and of these the most plausible which rapid resistance might develop is via the selection of resistance conferring alleles already present in the invasive carp population. By contrast, resistance development via *de novo* mutation would only occur over a large number of generations, a conclusion which was confirmed more formally by our second approach, i.e. forward population genetic modelling (Durr et al. 2020).

In this, the third study, we aimed to characterise the immunogenetics of invasive common carp in south-eastern Australia and to relate this to the considerable advances in the converse problem, i.e. breeding aquaculture strains of carp resistant to the CyHV-3. The most recent research on the latter has been detected four genomic loci (Tadmor-Levi et al. 2017), from which we were able to identify homologous common carp genes and use these as the basis for the SNP-based immunogenetic profiling of Australian and overseas carp populations to determine the extent to which sub-population structure might be identifiable.

In general, the immunogenetic profiling provided a patterning consistent with the conclusions from our literature review. Specifically, the goldfish and the goldfish hybrids formed very district clusters as would be expected from their experimentally confirmed high resistance to CyHV-3 (Hedrick et al. 2006; Yuasa et al. 2013). Regarding the carp strains, we were also able to identify a probable example of cryptic hybridisation of a Boolarra strain from an area in Victoria where this has been recorded in the past (Hume et al. 1983). Similarly, by comparison with the pure koi from Israel, evidence was presented that the feral koi in Sydney has undergone intra-specific hybridisation with common carp, and observation that is consistent with the conclusions made by Haynes et al. (2010) in a microsatellite-based population genetics study.

Whilst the immunogenetic profiling of the Australian and Israeli samples provided consistent results, this did not apply to all of the samples from the Czech Republic. In particular, one sample from an Amur scaly strain clustered very closely to a River Rhine strain (Figure 3b). Based on the examples of the cryptic hybridisation of the feral koi, such hybridisation between the aquaculture strains is a possible explanation for this apparent anomaly, as is the possibility that the sampled juvenile carp were misidentified. Potentially, these explanations could be resolved by examining further nuclear genes which enable strain identification (Xu et al. 2014). However, there is currently no published list of nuclear genes on which to base this strain identification and compiling and analysing these genes would require additional resource best handled as part of a follow-on study.

It is important to note that the evidence we present regarding the immunogenetic profile with respect to CyHV-3 resistance is indirect and relies on a number of assumptions including that the 29 selected innate immune genes truly determine relative resistance. A potential problem with this assumption is that it is based on a single QTL mapping study in which the immune genes were identified by homology to those of zebrafish (Tadmor-Levi et al. 2019b). As this is the most advanced study of its kind ever conducted, it does mean that we have made use of the most up-to-date data. Nevertheless, there are some inconsistencies

with other studies investigating resistance by carp to CyHV-3, including one which used the same Czech mirror carp strain that we sequenced (Palaiokostas et al. 2018).

A second assumption of the analysis – which clearly impacts our capability to make inference from our results - is that the 19 carp and goldfish hybrids sampled from the south-east Australia are representative of the entire population across the entire region. As part of our modelling work to develop a release strategy for CyHV-3, we estimated population sizes of adult and subadult common carp in five catchments across south-eastern Australia to be in the order of 50,000 to 5,000,000 per catchment. Clearly as viewed as a statistical sampling issue, this may seem an extremely serious limitation of our study. Nevertheless, a key finding of human population genomic studies over the past 30 years has been that through the careful selection of sub-populations, that the basic patterns of the genetic variability of the entire global population can be determined (Abecasis et al. 2012). Thus, although the number of carp genomes in our study is relatively small, we did manage to sample all the identified strains in the larger population genetics studies undertaken across south-eastern Australia using microsatellites (Haynes et al. 2009; Haynes et al. 2010; Haynes et al. 2012).

Whilst we consider our two assumptions to be reasonable, it would be clearly preferable if there was more direct evidence that invasive common carp in Australia do not possess the capability for rapid selection for resistance. The need for this is indicated from the infection trials undertaken to demonstrate that CyHV-3 does not affect non-target species showed considerable variation in the mortality of batches, varying between 50-100% (McColl et al. 2017). A naïve interpretation of this variable mortality based on simple Mendelian hereditary is that the surviving fish might have innate immunity to the virus, and the alleles responsible for this would then be selected. By contrast, the alternative hypothesis based on the aquaculture literature which shows that resistance to CyHV-3 is a complex polygenic trait, would lead us to expect that survival is determined to a large extent by chance and the interaction with other traits, such as those affecting general immune responsiveness to infection (Tadmor-Levi et al. 2019a). This presents a strong differentiating hypothesis for which we can use our immunogenetic PCA mapping to show that if hypothesis 1 is correct, then the survivors will be clearly differentiated from the susceptible. By contrast, if hypothesis 2 is correct, then there will be no differentiation. Of course, such a simple experiment will not definitely prove that surviving invasive common carp will or will not be rapidly selected for if the virus is released, but if it is shown to support hypothesis 2, then it will add to the weight-of-evidence that rapid resistance will not take place.

An important implication of our finding of an apparent strong immunogenetic profile for susceptibility to CyHV-3, is that the PCA mapping provides a method for monitoring the build-up of resistance post-release. What would be expected is that if resistance develops, then over time, the PCA mapping will show a gradual decrease in the proportion of carp with the susceptible SNP profile and an associated increase in a resistant one. This would however require a more cost-effective method to determine the immunogenetic SNP profile than the whole genome sequencing approach we used, viz at approximately $800 per sample. Such a more cost-effective approach would be to use “genotyping by sequencing” (GBS) which is now an established technique in aquaculture (Robledo et al. 2018) and conservation studies of freshwater fish populations (Couch et al. 2016).

Developing a post-release resistance monitoring system using GBS might even be extended to enable ongoing assessment of the effectiveness of the virus to reduce carp populations. Traditional methods for determining freshwater fish population sizes using the catch-per-unit-effort (CPUE) methods are expensive and labour intensive and are difficult to apply repeatedly across wide areas (Stuart et al. 2021). A recent innovation in fishery management has been the development of a SNP based method to estimate population size termed “close kin mark recapture” (CKMR) (Bravington et al. 2016b). This technique was originally introduced to estimate high-value pelagic fish populations such as blue-fin tuna (Bravington et al. 2016a), but has now been extended to freshwater fish (Ruzzante et al. 2019). Although further research would be needed to refine and validate CKMR for carp population estimation, the promise that through a single blood sample a simultaneous estimation of the build-up of resistance and population rebound (or not) might be possible argues strongly that this research should be a priority if a decision is made to release the virus.

Conclusion

Despite being highly exploratory, this study did manage to achieve its objectives in developing a transparent sequence-based method to assess genetic resistance to CyHV-3 and to use this to show that the immunogenetic profile of invasive common carp in Australia matches that of a well-characteristic aquaculture strain (Dor-70) and is distant to the relatively resistant strains derived from the Amur River sub-species of carp. While this result by itself does not prove that if the virus was released in Australia that rapid resistance to it will not develop, it does provide further evidence against this possibility that is consistent with our literature review and forward population genetic modelling.

Recommendations

As mentioned in the Discussion above, arising from this research we recommend two follow-on studies:

Study #1: Validation of SNP-based immunogenetics as a tool to assess susceptibility of invasive common carp to CyHV-3 challenge

- The objective of this study is to demonstrate that the SNP profile of survivors of experimental challenge of invasive common carp is no different than non-survivors as assessed through PCA mapping.
- The justification for this study arises from the need to show that the PCA immunogenetic approach is robust and valid before undertaking the more ambitious Study #2.
- If this study could make use of stored or ongoing challenge studies, this would be a very low-cost project, only requiring the genome sequencing to 8-10 carp. It would however require the implementation of more sophisticated bioinformatics analyses than undertaken in the present pilot study.

Study #2: Developing a post-release monitoring system to assess CyHV-3 field effectiveness

- The objective of this follow-on study is to develop and validate the use of genotyping-by-sequencing methods to simultaneous assess carp population size and the development of resistance to CyHV-3.
- The justification for this study is the presumed need to put into place a simple, cost-effective monitoring system to assess the field effectiveness of CyHV-3 as a biocontrol agent.
- This study would require a moderately sized budget, and thus would probably be conditional on a decision to release the virus. However, it might still be worthwhile undertaking some preliminary work to evaluate the CKMR method for estimating carp population size, as this would be useful irrespective of whether the virus is released or not.

Further development

See Study #1 above.

References

Abecasis GR, Auton A, Brooks LD, DePristo MA, Durbin RM, Handsaker RE, et al. (2012). An integrated map of genetic variation from 1,092 human genomes. *Nature* **491**:56-65.

Becker JA, Ward MP, and Hick PM (2019). An epidemiologic model of koi herpesvirus (KHV) biocontrol for carp in Australia. *Australian Zoologist* **40**:25-35.

Bolger AM, Lohse M, and Usadel B (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* **30**:2114-2120.

Boutier M, Donohoe O, Kopf RK, Humphries P, Becker J, Marshall JC, et al. (2019). Biocontrol of carp: the Australian plan does not stand up to a rational analysis of safety and efficacy. *Frontiers in microbiology* **10**:882.

Bravington MV, Grewe PM, and Davies CR (2016a). Absolute abundance of southern bluefin tuna estimated by close-kin mark-recapture. *Nature Communications* **7**:13162.

Bravington MV, Skaug HJ, and Anderson EC (2016b). Close-Kin Mark-Recapture. *Statistical Science* **31**:259-274.

Bushnell B (2014). BBMap: a fast, accurate, splice-aware aligner. Lawrence Berkeley National Lab. (LBNL), Berkeley, CA

Chang CC, Chow CC, Tellier LC, Vattikuti S, Purcell SM, and Lee JJ (2015). Second-generation PLINK: rising to the challenge of larger and richer datasets. *Gigascience* **4**:7.

Couch AJ, Unmack PJ, Dyer FJ, and Lintermans M (2016). Who's your mama? Riverine hybridisation of threatened freshwater Trout Cod and Murray Cod. *PeerJ* **4**:e2593.

Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, et al. (2013). STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* **29**:15-21.

Durr PA, Davis S, Joehnk K, Graham K, Hopf J, McColl KA, et al. (2019). Development of hydrological, ecological and epidemiological modelling to inform a CyHV3 release strategy for the biocontrol of carp in the Murray Darling Basin. Part A. Integrated ecological and epidemiological modelling. FRDC, Canberra.

Durr PA, Hopf J, Davis S, Samsing F, and Wynne JW (2020). Development of hydrological, ecological and epidemiological modelling to inform a CyHV3 release strategy for the biocontrol of carp in the Murray Darling Basin. Part B: Estimating the risk that carp will develop rapid genetic resistance to CyHV-3. FRDC, Canberra.

Haynes G, Gilligan D, Grewe P, Moran C, and Nicholas F (2010). Population genetics of invasive common carp *Cyprinus carpio* L. in coastal drainages in eastern Australia. *Journal of Fish Biology* **77**:1150-1157.

Haynes G, Gilligan D, Grewe P, and Nicholas F (2009). Population genetics and management units of invasive common carp *Cyprinus carpio* in the Murray–Darling Basin, Australia. *Journal of Fish Biology* **75**:295-320.

Haynes G, Gongora J, Gilligan D, Grewe P, Moran C, and Nicholas F (2012). Cryptic hybridization and introgression between invasive Cyprinid species *Cyprinus carpio* and *Carassius auratus* in Australia: implications for invasive species management. *Animal Conservation* **15**:83-94.

Hedrick RP, Waltzek TB, and McDowell TS (2006). Susceptibility of Koi Carp, Common Carp, Goldfish, and Goldfish × Common Carp Hybrids to Cyprinid Herpesvirus-2 and Herpesvirus-3. *Journal of Aquatic Animal Health* **18**:26-34.

Herrero J, Muffato M, Beal K, Fitzgerald S, Gordon L, Pignatelli M, et al. (2016). Ensembl comparative genomics resources. *Database* **2016**.

Hulak M, Kaspar V, Kohlmann K, Coward K, Tešitel J, Rodina M, et al. (2010). Microsatellite-based genetic diversity and differentiation of foreign common carp (*Cyprinus carpio*) strains farmed in the Czech Republic. *Aquaculture* **298**:194-201.

Hume D, Fletcher A, and Morison A (1983). Interspecific hybridization between carp (*Cyprinus carpio* L.) and golfish (*Carassius auratus* L.) from Victorian Waters. *Marine and Freshwater Research* **34**:915-919.

Jia Z, Wu N, Jiang X, Li H, Sun J, Shi M, et al. (2021). Integrative Transcriptomic Analysis Reveals the Immune Mechanism for a CyHV-3-Resistant Common Carp Strain. *Front Immunol* **12**:687151.

Kanehisa M, Furumichi M, Tanabe M, Sato Y, and Morishima K (2017). KEGG: new perspectives on genomes, pathways, diseases and drugs. *Nucleic Acids Res* **45**:D353-d361.

Koehn JD (2004). Carp (*Cyprinus carpio*) as a powerful invader in Australian waterways. *Freshwater Biology* **49**:882-894.

Kongchum P, Sandel E, Lutzky S, Hallerman EM, Hulata G, David L, et al. (2011). Association between IL-10a single nucleotide polymorphisms and resistance to cyprinid herpesvirus-3 infection in common carp (*Cyprinus carpio*). *Aquaculture* **315**:417-421.

Kopf RK, Boutier M, Finlayson CM, Hodges K, Humphries P, King A, et al. (2019). Biocontrol in Australia: Can a carp herpesvirus (CyHV-3) deliver safe and effective ecological restoration? *Biological Invasions* **21**:1857-1870.

Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, et al. (2009). The Sequence Alignment/Map format and SAMtools. *Bioinformatics* **25**:2078-2079.

Li JT, Hou GY, Kong XF, Li CY, Zeng JM, Li HD, et al. (2015). The fate of recent duplicated genes following a fourth-round whole genome duplication in a tetraploid fish, common carp (*Cyprinus carpio*). *Sci Rep* **5**:8199.

Marshall J, Davison AJ, Kopf RK, Boutier M, Stevenson P, and Vanderplasschen A (2018). Biocontrol of invasive carp: risks abound. *Science* **359**:877.

McColl KA, and Sunarto A (2020). Biocontrol of the Common Carp (Cyprinus carpio) in Australia: A Review and Future Directions. *Fishes* **5**.

McColl KA, Sunarto A, Slater J, Bell K, Asmus M, Fulton W, et al. (2017). Cyprinid herpesvirus 3 as a potential biological control agent for carp (*Cyprinus carpio*) in Australia: susceptibility of non-target species. *Journal of Fish Diseases* **40**:1141-1153.

Mintram KS, van Oosterhout C, and Lighten J (2021). Genetic variation in resistance and high fecundity impede viral biocontrol of invasive fish. *Journal of Applied Ecology* **58**:148-157.

Odegard J, Olesen I, Dixon P, Jeney Z, Nielsen H-M, Way K, et al. (2010). Genetic analysis of common carp (Cyprinus carpio) strains. II: Resistance to koi herpesvirus and Aeromonas hydrophila and their relationship with pond survival. *Aquaculture* **304**:7-13.

Palaiokostas C, Robledo D, Vesely T, Prchal M, Pokorova D, Piackova V, et al. (2018). Mapping and Sequencing of a Significant Quantitative Trait Locus Affecting Resistance to Koi Herpesvirus in Common Carp. *G3 (Bethesda)* **8**:3507-3513.

Palaiokostas C, Vesely T, Kocour M, Prchal M, Pokorova D, Piackova V, et al. (2019). Optimizing Genomic Prediction of Host Resistance to Koi Herpesvirus Disease in Carp. *Front Genet* **10**:543.

Rakus KŁ, Wiegertjes GF, Adamek M, Siwicki AK, Lepa A, and Irnazarow I (2009). Resistance of common carp (*Cyprinus carpio* L.) to Cyprinid herpesvirus-3 is influenced by major histocompatibility (MH) class II *B* gene polymorphism. *Fish & shellfish immunology* **26**:737-743.

Robledo D, Palaiokostas C, Bargelloni L, Martínez P, and Houston R (2018). Applications of genotyping by sequencing in aquaculture breeding and genetics. *Reviews in Aquaculture* **10**:670-682.

Ruzzante DE, McCracken GR, Førland B, MacMillan J, Notte D, Buhariwalla C, et al. (2019). Validation of close-kin mark–recapture (CKMR) methods for estimating population abundance. *Methods in Ecology and Evolution* **10**:1445-1453.

Samsing F, Hopf J, Davis S, Wynne JW, and Durr PA (2021). Will Australia's common carp (*Cyprinus carpio*) populations develop resistance to Cyprinid herpesvirus 3 (CyHV-3) if released as a biocontrol agent? Identification of pathways and knowledge gaps. *Biological Control* **157**:104571.

Seeb JE, Carvalho G, Hauser L, Naish K, Roberts S, and Seeb LW (2011). Single-nucleotide polymorphism (SNP) discovery and applications of SNP genotyping in nonmodel organisms. *Molecular Ecology Resources* **11**:1-8.

Shapira Y, Magen Y, Zak T, Kotler M, Hulata G, and Levavi-Sivan B (2005). Differential resistance to koi herpes virus (KHV)/carp interstitial nephritis and gill necrosis virus (CNGV) among common carp (*Cyprinus carpio* L.) strains and crossbreds. *Aquaculture* **245**:1-11.

Stuart IG, Fanson BG, Lyon JP, Stocks J, Brooks S, Norris A, et al. (2021). Continental threat: How many common carp (Cyprinus carpio) are there in Australia? *Biological Conservation* **254**:108942.

Tadmor-Levi R, Asoulin E, Hulata G, and David L (2017). Studying the Genetics of Resistance to CyHV-3 Disease Using Introgression from Feral to Cultured Common Carp Strains. *Front Genet* **8**:24.

Tadmor-Levi R, Doron-Faigenboim A, Marcos-Hadad E, Petit J, Hulata G, Forlenza M, et al. (2019a). Different transcriptional response between susceptible and resistant common carp (*Cyprinus carpio*) fish hints on the mechanism of CyHV-3 disease resistance. *BMC Genomics* **20**:1019.

Tadmor-Levi R, Hulata G, and David L (2019b). Multiple interacting QTLs affect disease challenge survival in common carp (*Cyprinus carpio*). *Heredity* **123**:565-578.

Wickham H (2016). *ggplot2: elegant graphics for data analysis (2nd ed)*. Springer, New York.

Wohlfarth G, Lahman M, Hulata G, and Moav R (1980). The story of "Dor-70", a selected strain of the Israeli common carp. *Bamidgeh* **32**:3-5.

Xu P, Zhang X, Wang X, Li J, Liu G, Kuang Y, et al. (2014). Genome sequence and genetic diversity of the common carp, *Cyprinus carpio*. *Nature genetics* **46**:1212-1219.

Yuasa K, Sano M, and Oseko N (2013). Goldfish is not a susceptible host of koi herpesvirus (KHV) disease. *Fish Pathology* **48**:52-55.

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