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Experimental transmission of infectious spleen and kidney necrosis virus (ISKNV) from freshwater ornamental fish to silver sweep *Scorpis lineolata*, an Australian marine fish

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ABSTRACT: The Australian native marine fish species, silver sweep *Scorpis lineolata*, is susceptible to the megalocytivirus *Infectious spleen and kidney necrosis virus* (strain DGIV-10) obtained from a freshwater ornamental fish, dwarf gourami *Trichogaster lalius*. This was demonstrated by direct inoculation and through cohabitation. Transmission by cohabitation was also demonstrated from inoculated freshwater Murray cod *Maccullochella peelii* to euryhaline Australian bass *Macquaria novemaculeata* and to marine silver sweep. The virus was also transmitted from infected marine silver sweep to euryhaline Australian bass and then to freshwater Murray cod. This study is the first to demonstrate the virulence of a megalocytivirus derived from ornamental fish in an Australian marine species and the first to show a feasible pathway for the exchange of megalocytiviruses between freshwater and marine finfish hosts. These results demonstrate that megalocytiviruses from freshwater ornamental fish have the potential to spread to diverse aquatic environments.

KEY WORDS: *Scorpis lineolata* · Megalocytivirus · Infectious spleen and kidney necrosis virus · Experimental transmission · Marine · Freshwater · Ornamental fish

1. INTRODUCTION

Megalocytiviruses affect a wide range of both freshwater and marine finfish species, but it is unclear whether megalocytivirus isolates from freshwater fish can be transferred naturally to fish in marine environments and vice versa. At a molecular phylogenetic level, based predominantly on major capsid protein (MCP) and, to a lesser degree, adenosine triphosphatase (ATPase) gene sequences, there is considerable similarity between some freshwater and marine megalocytivirus isolates. However, there has been a tendency to consider isolates of the infectious spleen and kidney necrosis virus (ISKNV) genotype as 'freshwater megalocytiviruses' and red seabream iridovirus (RSIV)- and turbot reddish body iridovirus (TRBIV)-like genotypes as 'marine megalocytiviruses' (Song et al. 2008, Fu et al. 2011). Nonetheless, there are examples of ISKNV-like megalocytiviruses associated with clinical disease outbreaks in marine fish such as grouper *Epinephelus* spp. and *Cromileptes altivelis* (Chao et al. 2002, Wang et al. 2007, Huang et al. 2011, Kurita & Nakajima 2012), large yellow croaker *Larimichthys crocea* (Wang et al. 2007), red drum *Sciaenops ocellatus* (Wang et al. 2007, Kurita & Nakajima 2012) and mullet *Mugil cephalus* (Kurita & Nakajima 2012). Conversely, there are examples of RSIV-like megalocytiviruses

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associated with disease outbreaks in freshwater mandarin fish *Siniperca chuatsi* (Fu et al. 2011, Dong et al. 2013) and marble goby *Oxyeleotris marmorata* (Huang et al. 2011, Chen et al. 2013).

TRBIV-like megalocytiviruses, which have recently been associated with disease outbreaks in marine fish such as barred knifejaw (also referred to as rock bream, beakperch and striped parrotfish) *Oplegnathus fasciatus*, have historically been associated with the live trade in ornamental fish in species as diverse as dwarf gouramis *Trichogaster lalius* (formerly *Colisa lalia*), three spot gouramis *Trichopodus trichopterus* (formerly *Trichogaster trichopterus*), freshwater angelfish *Pterophyllum scalare*, keyhole-cichlids *Cleithracara maronii* and oscars *Astronotus ocellatus* (Go et al. 2016, Koda et al. 2018). While some attempts have been made to experimentally transmit megalocytivirus isolates between different aquatic environments, such studies have been limited either by the use of direct (parenteral) inoculation of megalocytivirus (Dong et al. 2010) or by the separate evaluation of pathogenicity in either the marine or the freshwater environment, rather than as a continuum between the two (Jeong et al. 2008). While such studies may demonstrate the potential for a 'freshwater' megalocytivirus to infect a marine host or vice versa, they do not provide an indication of the mechanism by which such transmission might occur or whether there is potential for natural spread of megalocytiviruses between fish populations in these 2 different aquatic environments.

The aim of this study was to test the hypothesis that megalocytiviruses originating from ornamental fish could be spread from freshwater to marine environments, and vice versa, through the use of a euryhaline vector species.

2. MATERIALS AND METHODS

2.1. Species selection

Candidate marine species with potential susceptibility to infection with megalocytiviruses from freshwater ornamental fish were chosen following a literature review with consideration of the following criteria: (1) phylogenetic relationship to species with known susceptibility to megalocytiviruses, (2) presence in Australia and (3) availability for study.

Although ISKNV-like megalocytiviral DNA has been detected by PCR in apparently healthy individuals of many species of marine fish (Wang et al. 2007), clinical disease has been reported from fewer species (Table 1). A number of grouper species are particularly susceptible, and while Australia possesses many native species of grouper, including *Epinephelus malabaricus*, *E. coioides*, *E. lanceolatus* and *Cromileptes altivelis*, stock were not available for these trials. Barred knifejaw *Oplegnathus fasciatus* is highly susceptible to megalocytivirus infection (Jeong et al. 2008, Kurita & Nakajima 2012) but is not present in Australia. Although the genus *Oplegnathus* is monotypic within the family Oplegnathidae, studies undertaken using mitochondrial genomic data have indicated that the families Kyphosidae, Kuhliidae and, to a lesser extent, Terapontidae, have a strong phylogenetic relationship with the family Oplegnathidae (Yagishita et al. 2002, 2009). Silver sweep *Scorpis lineolata*, a member of the family Kyphosidae, are found in great abundance in shallow waters around reefs in New South Wales (NSW) (Edgar 2008) and are commonly captured by both commercial and recreational fishers in NSW (Stewart & Hughes 2005). Consequently, this species fulfilled all criteria and was selected as the marine species for use in the current study. Australian bass *Macquaria novemaculeata* was selected as a euryhaline vector (Go & Whittington 2019) and Murray cod *Maccullochella peelii* as a susceptible freshwater host (Go & Whittington 2006).

2.2. Source and maintenance of fish

Wild juvenile silver sweep, 25–90 mm total length (TL), were captured by handline from late winter to late summer during 2011 and 2012 from North Harbour in northern metropolitan Sydney (under NSW Department of Primary Industries scientific collection permit P12/0007-1.0). They were transferred to the University of Sydney Camden campus by road in aerated 15 l buckets. To reduce the potential parasitic burden, they were bathed in 200 ppm formalin for 1 h, and to minimise secondary bacterial infections as a consequence of capture and handling stress, they were then bathed in 100 mg l^{-1} oxytetracycline hydrochloride for 1 h (Noga 2000, Stewart & Hughes 2005). All silver sweep were initially housed in 400 l stock tanks with biological and mechanical filtration provided by a single canister filter (Model 405, Fluval) with supplementary aeration and maintained at a temperature of 25°C. Fish were generally quarantined in these stock tanks for a minimum of 4 wk prior to use in transmission trials. One group of silver sweep used in Experiment (Expt) 3B (see Section 2.3.3) was quarantined for 1 wk prior to use in that trial. Newly captured fish were fed with a diet of mar-

Table 1. Marine fish species reported to be clinically susceptible to infectious spleen and kidney necrosis virus (ISKNV)-like megalocytiviruses. Infection types were natural (N) and experimental (E; intraperitoneal inoculation and cohabitation). MCP: major capsid protein; VEGF: vascular endothelial growth factor; TNFR: tumor necrosis factor receptor

Species	Common name	Location	Mortality rate (%)	Date	Infection type	Technique used to determine ISKNV-like megalocytivirus involvement	Family	Reference
Cromileptes altivelis	Humpback grouper	Taiwan	40 (30–50)	2002	N	PCR typed (Cy15 amplicon)	Serranidae	Chao (2003)
		Indonesia	Not specified	2010	N	Phylogenetic analysis of MCP, ATPase, DNA polymerase genes and CY15, IRB6 amplicons		Murwantoko (2017)
Epinephelus awoara	Yellow grouper	China	60–80	2002	N	Phylogenetic analysis of MCP, VEGF, TNFR, mRNA (capping) genes	Serranidae	Wang et al. (2007)
E. coioides	Orange spotted grouper	China	60	2004	N	Phylogenetic analysis of MCP, ATPase gene sequences	Serranidae	Kurita & Nakajima (2012), NACA-FAO (2005)
		Indonesia	>80	<2009 (2001/2003?)	N	Phylogenetic analysis of MCP gene sequence		Murwantoko et al. (2009)
E. lanceo-latus	King grouper	Taiwan	60 (35–60)	2002	N	Sequencing of MCP (100% similar to ISKNV), ATPase (99% similar), CY15 (99% similar)	Serranidae	Chao (2003)
E. mala-baricus	Malabar grouper	China	60–80	2002	N	Phylogenetic analysis of MCP, VEGF, TNFR, mRNA (capping) genes	Serranidae	Wang et al. (2007)
Epinephelus hybrid	Hybrid grouper	Taiwan	60 (35–60)	1997	N	Sequencing of MCP (100% similar to ISKNV), ATPase (99% similar), CY15 (99% similar)/PCR typed (presumably over CY15 amplicon)	Serranidae	Chao (2003)
Larimichthys crocea	Large yellow croaker	China	60–80	2002	N	Phylogenetic analysis of MCP, VEGF, TNFR, mRNA (capping) genes	Sciaenidae	Wang et al. (2007)
Scatophagus argus	Spotted butterfish	Taiwan	5	2001	N	Sequencing of MCP (99% similar to ISKNV), ATPase (99% similar), CY15 (99% similar)	Scatophagidae	Chao (2003)
Sciaenops ocellatus	Red drum	China	60–80	1998, 2002	N	Phylogenetic analysis of MCP, VEGF, TNFR, mRNA (capping) genes	Sciaenidae	Wang et al. (2007), Yan et al. (2003)
Mugil cephalus	Flathead grey mullet	Singapore	10–80	1999/2000, 2003	N	Phylogenetic analysis of MCP and ATPase gene sequences	Mugilidae	Gibson-Kueh et al. (2004), Kurita & Nakajima (2012)
Oplegnathus fasciatus	Barred knifejaw	Korea	100	<2008	E	MCP sequence	Oplegnathidae	Jeong et al. (2008)

ine fish flakes (Wardley) before being weaned onto a proprietary aquaculture pellet food (1 mm aquafeed diet, Ridley). Based on the known biology of this species, which is believed to have a winter spawning season off the NSW coast, and combined with the available growth data (Stewart & Hughes 2005) and the size of the individuals collected, it was expected that all fish used in this trial were young-of-the-year that had been spawned the previous winter and recently settled in inshore areas (Fowler & Booth 2013); on this basis, the silver sweep were likely to be 2 to 3 mo old for Expt 1 and 8 to 10 mo old for Expts 2 and 3 (see Section 2.3).

Following quarantine, juvenile silver sweep were transitioned from 25 ± 1 to 28 ±1°C over 24 h and transferred to the experimental facility. The experimental facility and maintenance of fish was as described by Go & Whittington (2019), with the following exceptions: pH was between 7.8 and 8.2, and 35 ppt artificial seawater was made up from municipal tap water dechlorinated with a proprietary product (API Tapwater conditioner, Aquarium Pharmaceuticals) and a synthetic seawater mix (TetraMarine Salt Pro, Tetra). Temperature in each 100 l experimental tank (dimensions 50 × 50 × 40 cm) was maintained at 28 ± 1°C using a thermostatically controlled aquarium heater (Aquaone), consistent with temperatures known to be conducive to megalocytiviral infection (He et al. 2002, Go & Whittington 2006, Xu et al. 2008, 2010). Each experimental 100 l tank was maintained as a recirculating system with biological and mechanical filtration provided by a single hang-on filter (Aquaclear 110) containing shellgrit to assist in buffering capacity, filter wool and ceramic noodles. Water exchange was not performed unless required to maintain water quality.

Australian bass juveniles, <1.0 g, 25–35 mm TL and approximately 18 wk old, were obtained from a NSW commercial hatchery, with no previous history of megalocytivirus infection, in early February 2012. They were held in 100 l stock tanks at 25 ± 1°C until used in Expt 3 (see Section 2.3.3) in late April 2012, when fish were approximately 30 wk old and 40–50 mm TL. Australian bass were transitioned from 25 ± 1 to 28 ± 1°C over a period of 24 h prior to use in experiments at that temperature. In Expt 3A, they were transitioned to marine conditions (35 ppt salinity) by salinity adjustment at a rate of 5 ppt d^{-1} and held for at least 1 wk at marine conditions prior to use in experiments.

Murray cod juveniles, <2.0 g, 40–50 mm TL and approximately 18 wk old, were obtained from a separate NSW commercial hatchery with no previous history of megalocytivirus infection in mid-March 2012. They were held in 100 l stock tanks at a temperature of 25 ± 1°C until used in Expt 3 in late April, when the fish were approximately 24 wk old and 50–75mm TL. Experimental temperatures and acclimation from 25 ± 1 to 28 ± 1°C were as described above for silver sweep and Australian bass.

All fish used were considered free of megalocytiviruses on the basis that this group of viruses remains exotic to Australia in cultured food fish and in wild fish species (Go & Whittington 2006, Rimmer et al. 2015). Additionally, for all species, samples of kidney and/or splenic samples from all fish that died during quarantine were tested for megalocytivirus using quantitative real-time polymerase chain reaction (qPCR) to further verify the absence of megalocytivirus. In all cases, these samples tested negative for the presence of megalocytivirus.

2.3. Experimental design

Silver sweep were tested for susceptibility to *Infectious spleen and kidney necrosis virus*, strain DGIV-10, by intraperitoneal (IP) inoculation and cohabitation. Whether or not it was possible to transmit DGIV-10 from marine to freshwater environments and vice versa was then determined using silver sweep, Australian bass and Murray cod in the experimental model. Australian bass were used as the transfer host, being infected in either freshwater or marine conditions, and then transitioned to the reciprocal environment.

For all experiments, surviving fish were euthanised 28 d post exposure to DGIV-10. Exposure to DGIV-10 was defined to commence on the day of IP inoculation or on the day when fish were introduced into a tank for cohabitation of naïve fish with infected/exposed fish. The original source of the DGIV-10 inoculum was a single naturally infected dwarf gourami *Trichogaster lalius*, with DGIV-10 subsequently amplified in naïve Murray cod as described by Rimmer et al. (2017). The inoculation procedure followed that described by Go & Whittington (2019), but with anaesthesia in 35 ppt artificial seawater. The experimental design is summarised in Fig. 1.

2.3.1. Expt 1: Susceptibility of silver sweep to megalocytivirus by IP inoculation, pilot study

Megalocytivirus inoculum was prepared from the abdominal visceral homogenates of Murray cod

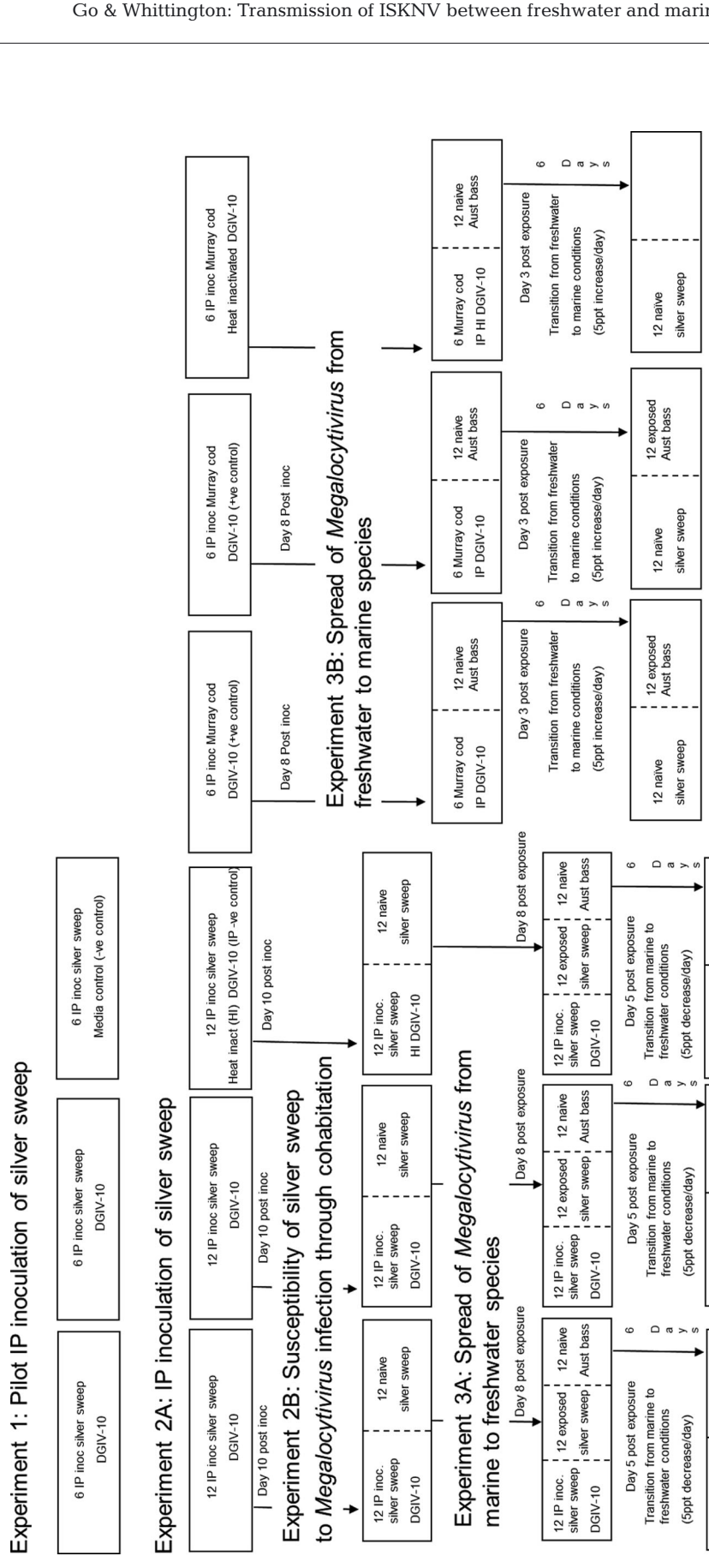

Fig. 1. Experimental design overview

experimentally inoculated with DGIV-10. Six juvenile silver sweep (25–30 mm TL; mean weight <1.0 g) were used in each group; fish in 2 groups were inoculated with 50 µL DGIV-10 inoculum (equivalent to 1×10^7 viral copies, as determined by qPCR based on a fragment of the MCP gene), while fish in one group of negative controls were injected with 50 µl homogenising medium. The groups were randomly allocated to tanks, and DGIV-10-inoculated fish were randomly allocated within treatments. As intraspecific aggression occurred in these small group sizes (with observations of extensive chasing and fin nipping of subdominant fish), larger group sizes (n = 12) were used in subsequent experiments.

2.3.2. Expt 2: Susceptibility of silver sweep to megalocytivirus by IP inoculation and cohabitation

Silver sweep, 75–90 mm TL, obtained in midsummer, were used in this experiment with 2 treatment groups and a single negative control group. Each group consisted of 12 silver sweep. Partitioned tanks were used as previously described by Go & Whittington (2019).

For Expt 2A, which further tested the susceptibility of silver sweep to megalocytivirus by IP injection; silver sweep were inoculated with 50 µl of DGIV-10 inoculum, while negative controls were inoculated with 50 µl of heat-inactivated DGIV-10 as described by Go & Whittington (2019). Murray cod that were concurrently inoculated in Expt 3B (see Section 2.3.3) served as positive controls for this experiment.

For Expt 2B, which tested the megalocytivirus susceptibility of silver sweep through cohabitation, 12 naïve silver sweep were introduced to the other side of the partitioned tank in each group on Day 10 post inoculation of the IP-injected fish.

2.3.3. Expt 3: Spread of megalocytivirus between marine and freshwater fish populations

Australian bass have previously been shown to be suitable as a megalocytivirus-susceptible model euryhaline vector species capable of transmitting megalocytiviral infection between different populations of freshwater fish (Go & Whittington 2019). This series of experiments was designed to test whether Australian bass could be used to transmit megalocytivirus infection from marine fish to freshwater fish and vice versa.

Expt 3A tested the transmission of megalocytivirus from marine fish to freshwater fish. Silver sweep in Expt 2 were used as viral-donor marine fish. On Day 18 post IP inoculation of the sweep in Expt 2 (i.e. Day 8 post introduction of the cohabiting naïve sweep), 12 naïve Australian bass, previously acclimated to marine conditions, were introduced into each of the 3 tanks (2 treatment tanks and a control tank) in nylon mesh aquarium fish net pens (dimensions: 16.5 × 12.5 × 13 cm, Hagen-Marina) on the side of the tank in which cohabiting silver sweep were held. These pens permitted the flow of water through the net without physical contact between Australian bass and silver sweep. The Australian bass were removed from the pens 5 d later, rinsed with fresh artificial seawater and transferred into one side of cleaned, disinfected, newly set up partitioned tanks adjusted to a salinity of 30 ppt. The salinity was reduced by 5 ppt d^{-1} by water exchange with reverse-osmosis water of the same temperature until freshwater conditions were achieved 6 d later. Twelve naïve Murray cod fingerlings, 50–75 mm TL and approximately 24 wk old, were then added to the other side of the partition in each tank. Fish were observed for 28 d from the time of introduction into each tank, and any surviving fish were sacrificed.

Expt 3B tested the transmission of megalocytivirus from freshwater fish to marine fish. Two groups of 6 Murray cod juveniles were injected with 50 µl of DGIV-10 inoculum, while 1 control group was given inactivated DGIV-10. Each group was placed on one side of a partitioned tank. On Day 8 post-inoculation, a group of 12 Australian bass was introduced to the other side of each tank. The Australian bass were removed after 3 d of exposure, rinsed with Milli-Q water and then transferred to new, cleaned and disinfected partitioned experimental tanks containing water at 5 ppt salinity. Salinity was increased at the rate of 5 ppt d^{-1} until 35 ppt marine conditions were achieved 6 d later. Groups of 12 naïve silver sweep, 75–90 mm TL and approximately 8 to 10 mo old were then placed on the other side of the partition in each tank. Fish were observed for 28 d from the time of introduction into each tank, and any surviving fish were sacrificed.

2.4. Sampling protocol

A single eye was excised from fish that had either died or had been subsequently euthanised. Each fish was rinsed in sterile distilled water and the surface of the cornea blotted with a clean sheet

of paper towel prior to the excision of the eye. A sterile, disposable scalpel blade was used to excise each eye, and a separate scalpel blade was used for each fish. The excised eyes were stored in 1.5 ml Eppendorf tubes at −80°C. The remainder of the fish was fixed in 10% neutral buffered formalin.

2.5. Preparation of tissue homogenates, DNA extraction and quantitative PCR analysis

Eyes were thawed immediately prior to use, and 9 volumes of homogenising medium (minimum essential medium) were added to each tube before homogenisation using a Fastprep system as described by Rimmer et al. (2012b). Briefly, each 2 ml homogenising tube contained 0.1 mm zirconia/silica beads (Daintree Scientific). Samples were processed in batches of 24 and were homogenised with the FastPrep system (MP Biosciences) for 40 s at a setting of 6.0 m s^{-1}. The resultant homogenate was clarified by centrifugation at 866 × *g* (10 min). A 50 µl aliquot of the supernatant was used for DNA extraction, using a MagMax-96 Viral Isolation Kit (Ambion) and a MagMax Express-96 particle processor (Applied Biosystems), as previously described by Rimmer et al. (2012b) with the modification that 50 µl of tissue homogenate were used to generate 70 µl of DNA eluate.

Quantitative PCR (qPCR) was performed in accordance with Rimmer et al. (2012a), using the primer pair C1073/C1074, targeting a 167 bp portion of the MCP gene. For quantification using this assay, a standard curve was produced by serial 10-fold dilution of plasmid containing a fragment of the MCP gene in molecular-grade water to produce template concentrations of 1×10^7 to 1×10^0 copies of MCP gene fragment per reaction. All reactions were run in duplicate, and a no-template control was included in each run. Quantification of viral DNA was interpolated from the plasmid DNA standard curve, based on the threshold cycle number (Ct) determined after 40 cycles, of the unknown sample. Details of qPCR interpretation for this assay are described by Go & Whittington (2019).

2.6. Statistical analyses

Final mortality rates between control and treatment groups were compared using Fisher's exact test applied to a 2 × 2 contingency table where the variable analysed was status at end of trial (i.e. a comparison of the proportions 'live' vs. 'dead') for each group with a significance threshold of $p < 0.05$ (http://graphpad.com/quickcalcs/contingency1.cfm).

2.7. Histopathology

Representative fish from each experiment were examined (see Table 2). *In situ* hybridisation (ISH) was also undertaken on a single individual from Expt 1 to verify that lesions consistent with published descriptions of megalocytivirus-associated pathology contained megalocytiviral genetic material. ISH was performed as described by Go et al. (2016), using a polynucleotide probe generated by primer pair C1330/C1331. Whole fish, with an eye removed, were fixed for a period of 48 to 72 h in 10% neutral buffered formalin after opening the abdominal cavity. Each fish was decalcified in 125 g l^{-1} EDTA (Sigma-Aldrich) in distilled water for 3 d (Roberts 2012) to several months before processing (most common duration was 3–4 wk). Small fish were sectioned longitudinally and placed directly into the cassette, whereas for larger specimens, abdominal viscera, including spleen, liver and intestine, were dissected, and a section of posterior kidney was removed in transverse section with associated spinal musculature. Where possible, an eye, gill and anterior kidney were also included.

3. RESULTS

3.1. Experimental transmission

A summary of mortality, Ct, viral genome copy number (based on copies of the MCP gene fragment) and status at the end of the trial ('live' or 'dead') is presented in Table 2. A more detailed description for individual experiments is presented below.

3.1.1. Expt 1: Susceptibility of silver sweep to megalocytivirus by IP inoculation, pilot study

Intraspecific aggression occurred in all 3 tanks. However, clinical signs of inappetence, depigmentation and tachybranchia consistent with megalocytiviral infection were only observed in silver sweep in the 2 treatment tanks, and mortality occurred the day after the clinical signs began on Day 9 post inoculation. Two mortalities were observed in the control tank, although severe intraspecific aggression was observed and the dead fish had severely frayed fins.

Table 2. Summary of results from experimental transmission trials. For calculation of mean threshold cycle number (Ct), samples yielding no Ct were ascribed an arbitrary Ct of 41. NAD: no abnormalities detected; IBC: inclusion body bearing cell. For experiment 3B, results for individual tank treatment groups (1 and 2) are shown

Expt	Group description	n	End of trial outcome	n	— qPCR — +ve	-ve	Ct (mean ± SD)	Viral genome copy number (mean ± SD) mg^{-1}	Mortality rate (%)	Histological examination — n	n with IBCs	Presence of *Amyloodinium*-like organism	Summary of findings
1	DGIV-10 IP-inoculated silver sweep	12	Live	5	3	2	36.41± 4.31	$5.36 \pm 6.16 \times 10^{2}$	58	2	0	0	NAD
			Dead	7	7	0	16.16 ± 4.03	$4.53 \pm 9.55 \times 10^{9}$		3	3	0	Abundant IBCs in spleen and intestinal lamina propria and to a lesser extent, liver
	Media IP-inoculated silver sweep	6	Live	4	0	4	No Ct	0	33	1	0	0	NAD
			Dead	2	0	2	No Ct	0		1	0	0	NAD
2A	DGIV-10 IP-inoculated Murray cod (positive control)	12	Live	0	–	–	–	–	100				
			Dead	12	11	1	19.65 ± 6.82	$8.15 \pm 7.10 \times 10^{7}$		5	5	0	Sporadic IBC presence, particularly in spleen and kidney and occasionally liver
	DGIV-10 IP-inoculated silver sweep	24	Live	9	5	4	37.00 ± 4.19	$2.08 \pm 3.93 \times 10^{3}$	63	3	0	0	NAD
			Dead	15	15	0	20.05 ± 1.97	$2.97 \pm 3.79 \times 10^{7}$		6	6	0	Abundant IBCs in most individuals, particularly in spleen, intestinal lamina propria and kidney and to a lesser extent, liver
	Heat inactivated DGIV-10 IP-inoculated silver sweep (negative control)	12	Live	12	0	12	No Ct	0	0	2	0	0	NAD
			Dead	0	–	–	–	–					
	Heat inactivated DGIV-10 IP-inoculated Murray cod (negative control)	6	Live	12	0	6	No Ct	0	0	2	0	0	NAD
			Dead	0	–	–	–	–					
2B	Silver sweep cohabitated with IP-infected silver sweep	24	Live	0	–	–	–	–	100				
			Dead	24	22	2	20.63 ± 6.71	$8.08 \pm 13.6 \times 10^{7}$		7	7	0	Abundant IBCs in most individuals particularly in spleen, intestinal lamina propria and kidney and to a lesser extent, liver
	Silver sweep cohabitated with heat-inactivated DGIV-10 silver sweep	12	Live	12	0	12	No Ct	0	0	2	0	0	NAD
			Dead	0	–	–	–	–					
3A	Australian bass cohabitated with silver sweep exposed to DGIV-10 by cohabitation	24	Live	0	–	–	–	–	100				
			Dead	24	24	0	16.44 ± 1.77	$2.17 \pm 2.39 \times 10^{8}$		7	7	0	Sporadic IBC presence, particularly in spleen and kidney and occasionally liver
	Australian bass cohabitated with silver sweep exposed to heat-inactivated DGIV-10 (negative control)	12	Live	12	0	12	No Ct	0	0	2	0	0	NAD
			Dead	0	–	–	–	–					

Table 2. (continued)

Expt	Group description	n	End of trial outcome	n	qPCR +ve	qPCR -ve	Ct (mean ± SD)	Viral genome copy number (mean ± SD) mg^{-1}	Mortality rate (%)	Histological examination: n	Histological examination: n with IBCs	Histological examination: Presence of *Amyloodinium*-like organism	Histological examination: Summary of findings
Murray cod	24 cohabitated with Australian bass exposed to DGIV-10 by cohabitation	Live	0	–	–	–	–	100					
			Dead	24	24	0	17.59 ± 1.96	9.21 ± 10.8 × 10^7		6	6	0	Sporadic IBC presence, particularly in spleen and kidney and occasionally liver
	Murray cod cohabitated with Australian bass exposed to heat-inactivated DGIV-10 by cohabitation	12	Live	12	0	12	No Ct	0	0	2	0	0	NAD
			Dead	0	–	–	–	–					
3B	Australian bass cohabitated with DGIV-10 IP-inoculated Murray cod-1	12	Live	0	–	–	–	–	100				
			Dead	12	12	0	18.55 ± 2.73	9.10 ± 8.40 × 10^7		2	2	0	Sporadic IBC presence, particularly in spleen and kidney and occasionally liver
	Australian bass cohabitated with DGIV-10 IP-inoculated Murray cod-2	12	Live	0	–	–	–	–	100				
			Dead	12	9	3	32.07 ± 9.27	5.73 ± 10.7 × 10^6		3	0	3	Lamellar hyperplasia associated with abundant *Amyloodinium*-like organisms; NAD in other organs
	Australian bass cohabitated with heat-inactivated DGIV-10 IP-inoculated Murray cod (negative control)	12	Live	12	0	12	No Ct	0	100	2	0	0	NAD
			Dead	0	–	–	–	–					
	Silver sweep cohabitated with Australian bass exposed to DGIV-10 by cohabitation-1	12	Live	1	1	0	37.82	1.06 × 10^2	92	1	0	0	NAD
			Dead	11	11	0	21.10 ± 5.44	5.31 ± 6.42 × 10^7		3	3	0	Abundant IBCs in most individuals, particularly in spleen, intestinal amina propria and kidney and to a lesser extent, liver
												1	
	Silver sweep cohabitated with Australian bass exposed to DGIV-10 by cohabitation-2	12	Live	0	–	–	–	–	100				
			Dead	12	11	1	27.68 ± 5.23	1.10 ± 3.54 × 10^6		2	0	2	Lamellar hyperplasia associated with abundant *Amyloodinium*-like organisms; NAD in other organs
	Silver sweep cohabitated with Australian bass exposed to heat-inactivated DGIV-10 by cohabitation (negative control)	12	Live	12	0	12	No Ct	0	0	2	0	0	NAD
			Dead	0	–	–	–	–					

All mortalities from the treatment tanks were positive by qPCR with viral copy numbers (based on copies of the MCP gene fragment) of 2.59×10^{10} to 1.06×10^{7} mg^{-1} tissue, and 3 of 5 surviving fish had 1.50×10^{3} to 5.25×10^{2} viral copies mg^{-1} tissue. None of the control fish tested positive.

3.1.2. Expt 2: Susceptibility of silver sweep to megalocytivirus by IP inoculation and cohabitation

In Expt 2A, which tested the susceptibility of silver sweep by IP inoculation, clinical signs of inappetence, tachybranchia and pigmentation changes were first observed in IP-inoculated silver sweep on Day 9 post inoculation in both treatment tanks, whereas no clinical signs were observed in the controls inoculated with heat-inactivated DGIV-10. Severe morbidity, defined by loss of equilibrium, lateral recumbency and non-responsiveness to stimulation with a net, immediately preceded mortality. Mortalities commenced on Days 9 and 10 post inoculation in treatment tanks, and there was an overall mortality rate of 62.5% within 17 d post inoculation. The remaining fish in treatment tanks and the control group did not show any clinical signs and survived until they were sacrificed on Day 28 post inoculation. All silver sweep that died during the trial tested positive by qPCR with viral copy numbers (based on copies of the MCP gene fragment) of 1.14×10^{8} to 2.60×10^{6} per mg tissue. Additionally, of the 9 surviving sweep from the treatment groups that were sacrificed at the termination of the trial, 5 had viral copy numbers of 4.31×10^{3} to 1.57×10^{2} per mg tissue. In contrast, none of the control IP-inoculated sweep tested positive by qPCR.

In Expt 2B, which tested the susceptibility of silver sweep to megalocytivirus infection through cohabitation, a single mortality was observed on the 1st day after exposure in 1 treatment tank. Additional mortalities were observed on Days 3 and 6, prior to the onset of clinical signs. These fish had signs of intraspecific aggression, namely tattered fins and loss of scales. Commencing on Days 7 and 9 post exposure in each treatment tank, inappetence, pale pigmentation change and tachybranchia were observed. However, due to the active swimming habits of this species, accurate assessment of the numbers of fish exhibiting clinical signs was difficult. Mortalities commenced in the 2 treatment tanks on Days 8 and 10 post exposure, and all fish died within 13 d post exposure. No mortalities were observed in the controls throughout the 28 d trial. Quantitative PCR tests were negative for fish that died prior to Day 6. However, the silver sweep that died on Day 6 post exposure had a viral copy number of 4.15×10^{5} copies mg^{-1}. All remaining silver sweep mortalities tested had viral copy numbers of 6.10×10^{8} to 3.87×10^{6} mg^{-1} tissue. None of the control silver sweep tested positive for megalocytiviral DNA by qPCR.

3.1.3. Expt 3: Spread of megalocytivirus between marine and freshwater fish populations

Expt 3A tested the transmission of megalocytivirus from marine fish to freshwater fish. Australian bass cohabitated with silver sweep that had been exposed to DGIV-10 began to exhibit clinical signs of inappetence and tachybranchia on Day 11 post exposure, and mortality was first observed on the following day. Some clinically affected Australian bass had long white faecal casts. Most of the exposed Australian bass died within 3 wk of exposure to DGIV-10, 1 fish died on Day 22, and the last Australian bass was moribund at the end of this component of the trial 28 d post exposure. All Australian bass in the treatment groups tested strongly positive by qPCR, with 4.42×10^{8} to 3.32×10^{6} viral copies (based on copies of the MCP gene fragment) mg^{-1} tissue. In contrast, none of the Australian bass in the control group died, and none tested positive for megalocytiviral DNA by qPCR.

The treatment groups of Murray cod that, in turn, were exposed to the Australian bass began to exhibit clinical signs of inappetence, tachybranchia and dark discolouration on Day 9 post exposure, and all were dead by Day 16 post exposure. All Murray cod in the treatment tanks tested positive by qPCR, with 1.98×10^{8} to 3.85×10^{6} viral copies mg^{-1} tissue. In contrast, no mortalities were observed in the control tank, and none of the fish tested positive for megalocytiviral DNA by qPCR.

Expt 3B tested the transmission of megalocytivirus from freshwater fish to marine fish. One of the Murray cod that had been IP-inoculated with DGIV-10 died on Day 8 post inoculation, with signs of intraspecific aggression and cannibalism, and was qPCR negative. However, a number of others had tachybranchia and inappetence on Day 8, and mortalities were observed on Day 9. By Day 20 post inoculation, all Murray cod in the treatment tanks were dead and all tested positive for megalocytiviral DNA, with viral copy numbers (based on copies of the MCP gene

fragment) of 2.35×10^8 to 1.68×10^7 mg^{-1} tissue. Australian bass which were cohabitated with these Murray cod in turn began to die on Day 9 post-exposure, and all were dead by Day 14 post-exposure. In one tank, all mortalities tested positive by qPCR, with viral copy numbers of 2.38×10^8 to 1.90×10^6 mg^{-1} tissue. In the other tank (no. 2; see Table 2), 9 of the 12 Australian bass tested positive for megalocytiviral DNA, with viral copy numbers of 2.94×10^7 to 9.32×10^0 mg^{-1} tissue (only 4 fish yielded copy numbers greater than 5×10^5 mg^{-1} tissue). Mortalities in silver sweep that were in turn cohabitated with these Australian bass were first observed on Day 7 post exposure, and, at the termination of the trial 28 d post exposure, only 1 surviving fish remained. In Tank 1, silver sweep had tachybranchia and pigmentary change, whereas in Tank 2 only tachybranchia was observed. In Tank 1, all fish tested positive for megalocytiviral DNA by qPCR, with 10/12 fish producing copy numbers in excess of 5×10^5 mg^{-1} tissue, whereas in Tank 2, qPCR results were variable, with 11/12 silver sweep testing positive by qPCR, but only 1 single silver sweep yielding a copy number greater than 5×10^5 mg^{-1} tissue and 9/12 silver sweep yielding copy numbers greater than 1×10^4 mg^{-1} tissue.

3.2. Comparison of mortality rates

Mortality rates are shown in Fig. 2. Plots for each group represent data pooled data for both tanks, with the exception of Expt 3B, in which only silver sweep and Australian bass results from Tank 1 are presented. The difference in final mortality rates between control and treatment groups were statistically significant for all experiments ($p < 0.001$) except Expt 1 ($p = 0.6199$), in which group sizes were low and signs of intraspecific aggression were observed.

3.3. Gross pathology

On necropsy, fish from the treatment groups demonstrated anaemia evident as gill pallor and had splenomegaly and an enlarged gall bladder when compared with fish from control groups (Fig. 3).

3.4. Histopathology and ISH

Inclusion body bearing cells (IBCs), consistent with previous descriptions of megalocytiviral infection (Sudthongkong et al. 2002), were observed in all treatment groups of fish, particularly in the spleen, the lamina propria of the intestines, the anterior and posterior kidney, the choroid of the eye and, to a lesser extent, the liver and the gills (Fig. 4). These changes were observed in all treatment groups, except Tank 2 in Expt 3B. All 5 fish examined from this tank (3 Australian bass and 2 silver sweep) had severe lamellar hyperplasia and infiltration, and protozoal parasites consistent in appearance with *Amyloodinium ocellatum* were observed in close association with the gill lamellae (not shown); IBCs were not observed. Neither lamellar hyperplasia and infiltration nor *A. ocellatum* were observed in samples from any other tank. Silver sweep had large numbers of IBCs particularly in the spleen and the lamina propria of the intestines. Using DIG-ISH on samples from a representative fish, some IBCs were shown to contain megalocytiviral DNA (Fig. 5) which stained dark purple-black, although many other adjacent IBCs remained unstained.

4. DISCUSSION

This study is the first to demonstrate transmission of megalocytivirus between freshwater and marine fish, in both directions, via a natural route of exposure. It is also the first report of an Australian marine fish species that is susceptible to a megalocytivirus isolate derived from ornamental fish. Although a previous study has shown that an ISKNV-like megalocytivirus, obtained from pearl gouramis *Trichopodus* (*Trichogaster*) *leerii* (freshwater), could infect barred knifejaw (marine) via direct inoculation (Jeong et al. 2008), it did not demonstrate how a megalocytivirus derived from a freshwater ornamental fish species could be spread to a marine species. The findings from the current study suggest that spread in water between freshwater and marine aquatic ecosystems is possible via infection of a megalocytivirus-susceptible euryhaline species, in this case, Australian bass. This scenario mimics what might occur in the natural environment and suggests a plausible mechanism for pan-aquatic dispersal of megalocytiviruses.

This series of experiments demonstrated that silver sweep are highly susceptible to an ISKNV-like megalocytivirus by both IP and cohabitation routes and re-confirmed the susceptibility of Australian bass and Murray cod. Susceptibility determination in all 3 foodfish species to DGIV-10, which was derived from ornamental fish, was consistent with the criteria defined by the World Organisation for Animal Health (OIE 2018), whereby susceptibility was demonstrated

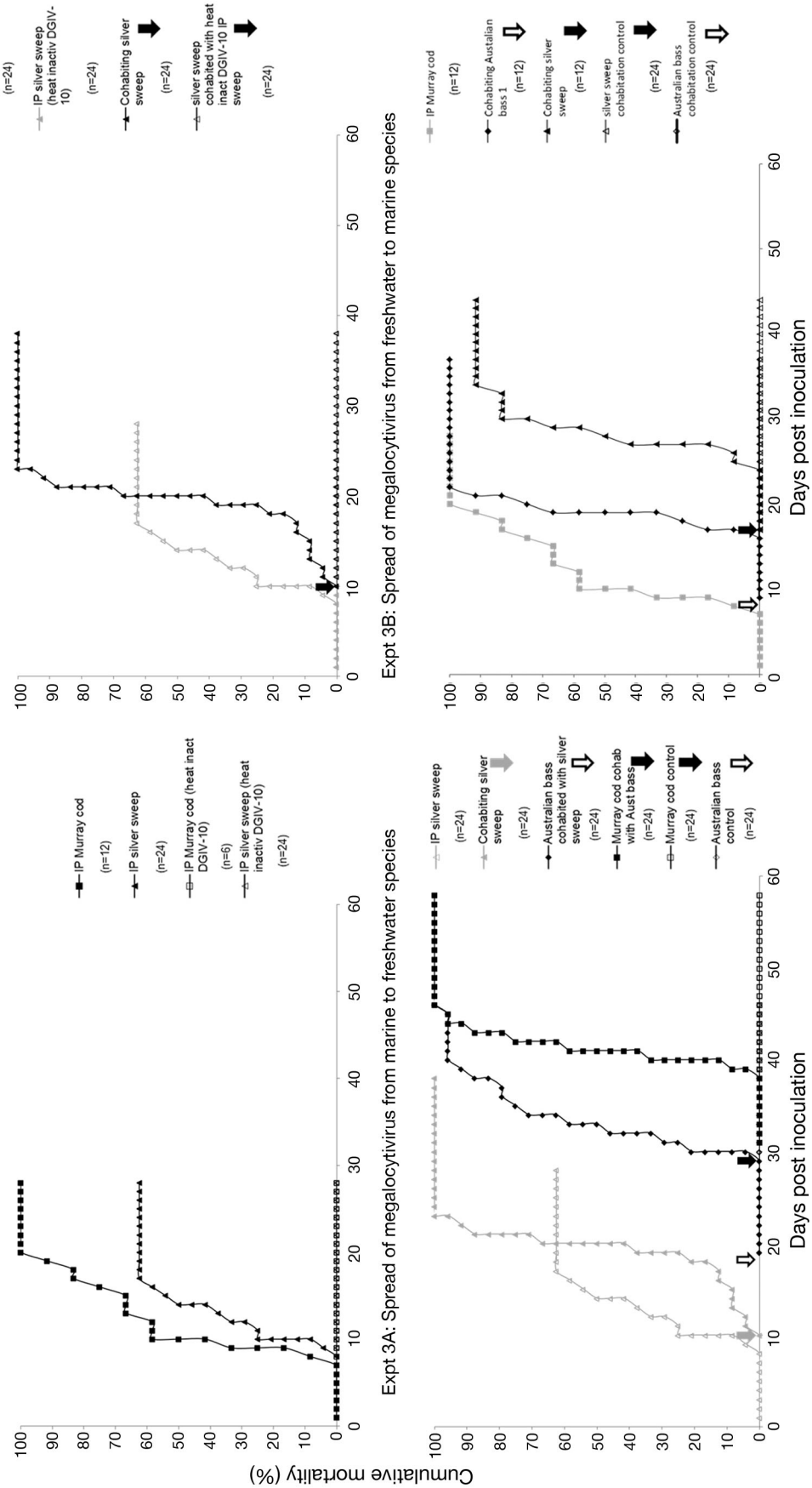

Fig. 2. Cumulative mortality for Expts 2 and 3. For cohabitation experiments, cumulative mortalities of infected donor fish are shown in grey. Numbers in parentheses indicate number of fish used to calculate mortality rates, and arrows indicate time of introduction of cohabitating fish. Mortality data for Expt 3B are for Tank 1 only, due to confounding effects of *Amyloodinium* infection in Tank 2

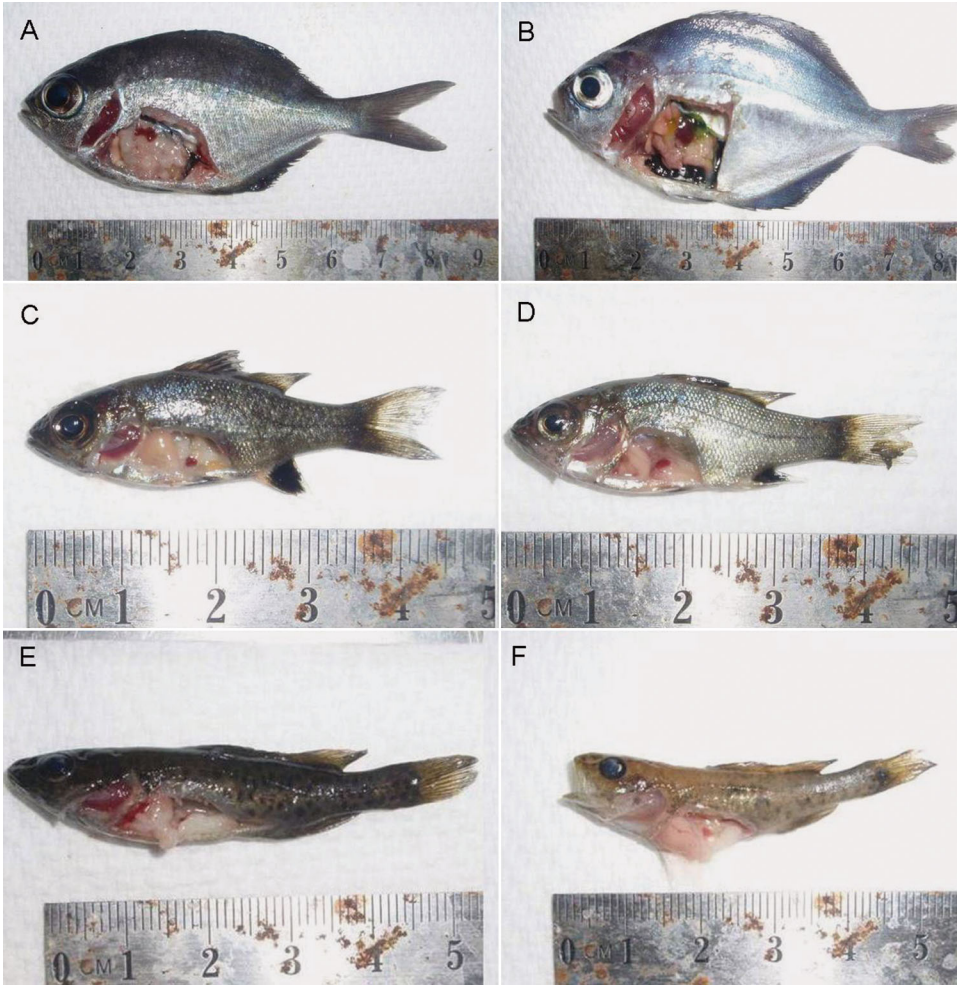

Fig. 3. Comparison of control (left column) and megalocytivirus-affected fish (right column): (A,B) silver sweep; (C,D) Australian bass; (E,F) Murray cod infected through cohabitation. In E, a haemorrhage at the cranial margin of the abdominal cavity is a dissection artefact. Gill pallor, general epidermal pallor and splenomegaly are present in the affected specimens (B,D, F), but not in the control unaffected fishes (A,C,E). A distended gall bladder can also be seen in the affected silver sweep (B)

through transmission consistent with a natural pathway (cohabitation), the pathogenic agent was characterised through qPCR, and true infection was demonstrated through histopathology and ISH. The demonstration of megalocytiviral DNA within IBCs using ISH provided clear evidence that these lesions were the result of infection with a megalocytivirus, consistent with findings of previous megalocytivirus experimental challenge studies (Chao et al. 2004, Go & Whittington 2019). IBCs were observed in 3 fish that tested positive by qPCR, and many IBCs from one of these fish stained strongly positive by ISH. However, it is unclear why many adjacent cells, which appeared to be similar, did not stain positively using ISH. It is possible that the lack of staining was due to low viral DNA quantity, perhaps due to different stages of viral replication.

Affected silver sweep had clinical signs of tachybranchia, inappetence and epidermal pigmentary changes, all of which have been reported in megalocytivirus infection in other species (He et al. 2000, Chen et al. 2003, Lancaster et al. 2003, Go & Whittington 2006). Clinical signs in Australian bass, which were also susceptible, were consistent with those reported in other species, with the additional observation of a trailing white faecal cast in some fish. This has also been reported in Banggai cardinalfish *Pterapogon kauderni* infected with an ISKNV-like megalocytivirus (Weber et al. 2009), and it would be interesting to determine if this clinical sign is more widespread in cases of megalocytiviral infection, but under-reported due to the difficulty of very close observation.

Silver sweep appeared to be more susceptible to DGIV-10 via cohabitation than by direct IP inoculation, and lesions typically associated with megalocytiviral infection in IP inoculated fish were generally less severe compared to those infected via cohabitation. This was surprising because IP inoculation is usually considered to be a more direct route of inoculation, able to bypass natural barriers to infection (Hoar et al. 1997). Although

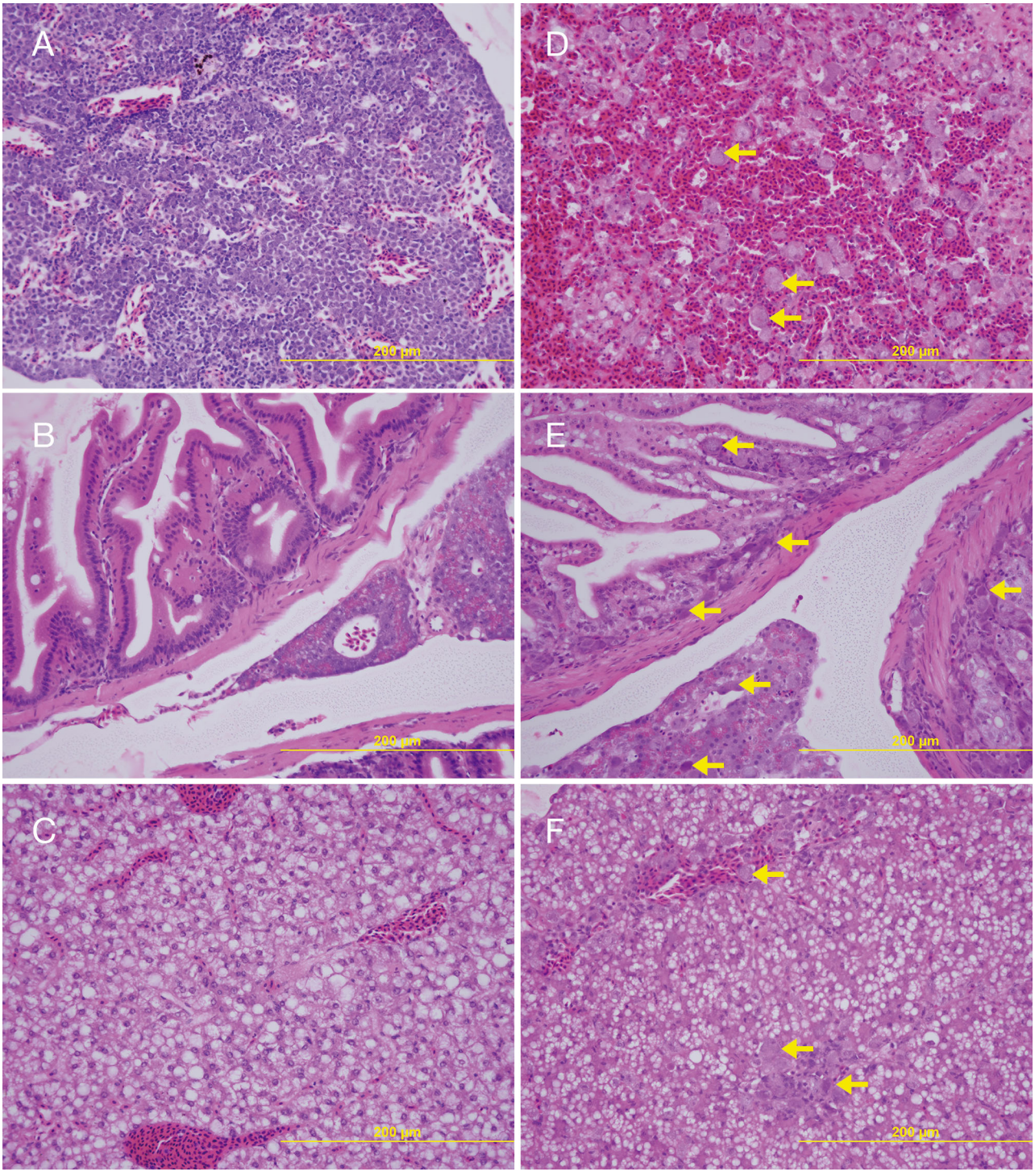

Fig. 4. Comparison of H&E-stained histological sections derived from (A–C) clinically normal control silver sweep and (D–F) silver sweep cohabitated with Australian bass infected by cohabitation with Murray cod inoculated with DGIV-10. Sections of spleen (A,D), intestine and pancreatic tissue (B,E) and liver (C,F) are shown. Representative examples of inclusion body bearing cells (IBCs) are indicated by yellow arrows. Disruption to splenic architecture by IBCs can be observed (D). IBCs are also abundant in the intestinal lamina propria (E) and around the hepatic vessels (F)

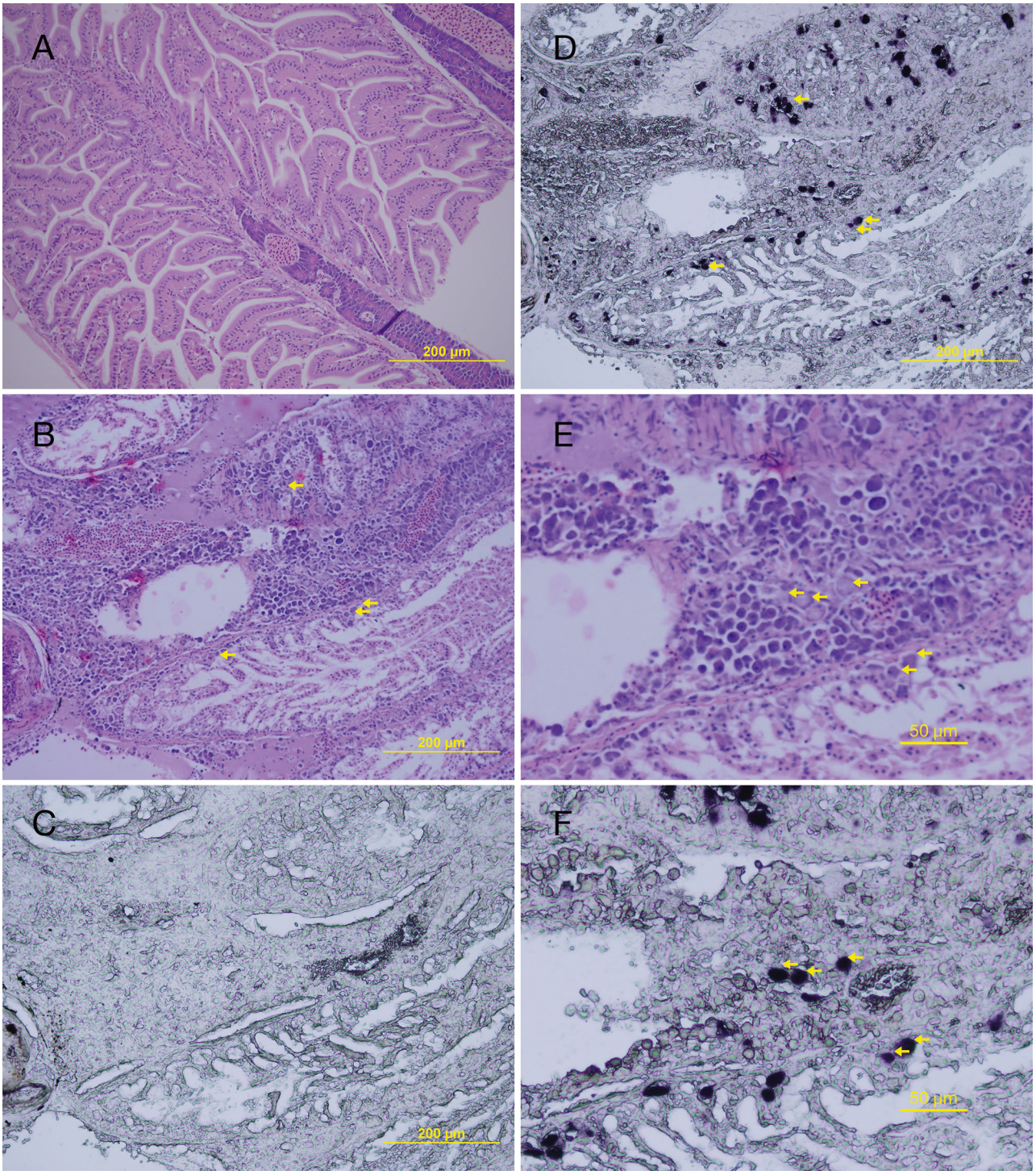

Fig. 5. Serial sections of intestinal epithelium from (A) unaffected control silver sweep compared with affected silver sweep (identification: SVC 11/162-A1) from Expt 1 with IBCs stained by (B) H&E, (C) *in situ* hybridisation (ISH) in the absence of probe (negative control) and (D) ISH in the presence of polynucleotide probe generated by primer pair C1330/C1331. ISH slides were not counter-stained. (E,F) Detailed insets of B and D, respectively. Representative IBCs stained both by H&E and ISH are indicated by yellow arrows. Melanin in no-probe controls appeared brown-black compared with blue-black positive staining. In the affected tissue (B–F), there are abundant IBCs causing disruption to, and in some areas replacement of, the normal intestinal epithelial architecture

the basis for the decreased mortality rate observed in silver sweep challenged by IP inoculation is unknown, a similar observation was noted by Mahardika et al. (2004) in humpback grouper *Cromileptes altivelis* in which a primary tissue filtrate containing the megalocytivirus grouper sleepy disease iridovirus induced a lower rate of mortality compared to the same filtrate diluted to 10^{-4}. In that study, it was suggested that a protective interferon-like substance was present in the undiluted injected homogenate which resulted in lower rates of mortality than in diluted filtrate.

In Expt 1, intraspecific aggression was observed in all tanks and is likely to have directly accounted for the mortality observed in the control group. This aggression may have occurred as a result of the small numbers of fish in each tank. In studies involving the closely related blue mao mao *Scorpis violaceus*, Lowe & Wells (1996) found that a density of 15 ind. $tank^{-1}$ was critical in ensuring normal feeding and schooling behaviour. In Expts 2 and 3, we used a density of 12 silver sweep $tank^{-1}$, and intraspecific aggression was reduced.

Successful transmission of an ISKNV-like megalocytivirus was confirmed through qPCR in all treatment replicates in all experiments. Fish tested strongly positive for megalocytiviral DNA by qPCR, with the majority producing copy numbers (based on copies of the MCP gene fragment) greater than 1×10^6 copies mg^{-1} tissue, consistent with other studies (Wang et al. 2006, Jun et al. 2009, Wu et al. 2009), demonstrating the value of qPCR in determining whether a fish is likely to have a clinically significant megalocytiviral infection. However, in 1 treatment tank in Expt 3B, severe gill disease due to *Amyloodinium ocellatum* affected both silver sweep and Australian bass and compromised outcomes related to the viral infection. There was a shortage of silver sweep at the time, and so this tank had been populated using silver sweep that had been quarantined for only 1 wk, compared with a minimum of 4 wk in other trials, to allow sufficient time to express disease associated with pathogens that survived the formalin and oxytetracycline bath. However, megalocytiviral DNA was still detected in many of these fish at the time of mortality. Consequently, the silver sweep and the Australian bass in this particular tank are likely to have died with megalocytiviral infection, rather than as a result of that infection.

The use of heat-inactivated DGIV-10 negative controls ensured that an accurate comparison could be made between treatment and control groups. While the vast majority of IP or intramuscular studies of megalocytivirus transmission have used tissue homogenates from infected fish and homogenising media or sterile PBS as controls (Sano et al. 2001, Mahardika et al. 2004, Jeong et al. 2006, 2008, Jun et al. 2009), such controls do not accurately simulate the inoculation of strongly PCR-positive megalocytiviral material derived from moribund or dead fish. There is potential for such material to elicit a host reaction, and potential for the presence of megalocytiviral DNA in this initial inoculum to confound PCR test results, particularly if such material is inoculated into the IP cavity, and abdominal organs are subsequently sampled. The use of a heat-inactivated tissue homogenate can address many of these issues and facilitate a better comparison between active viral replication in a susceptible test species compared with no viral replication in a non-susceptible species. However, if there are heat labile factors within the viable homogenate with the potential to induce a host response in the inoculated fish, then these will be absent in the heat-inactivated inocula.

The susceptibility of silver sweep to DGIV-10 supports the assumption that related species may be susceptible to megalocytiviral infection. For example, members of the family Percichthyidae, including mandarin fish *Siniperca chuatsi* (He et al. 2002), leopard mandarin fish *S. scherzeri* (Shin et al. 2014), Murray cod (Lancaster et al. 2003, Go & Whittington 2006), golden perch *Macquaria ambigua*, Macquarie perch *M. australasica* (Rimmer et al. 2017) and Australian bass (Go & Whittington 2019), are all clinically susceptible to infection with ISKNV-like megalocytiviruses. Interestingly, in experimental transmission trials, of the range of fish species tested, only mandarin fish and largemouth bass *Micropterus salmoides* were demonstrated to be clinically susceptible to the type strain of ISKNV (He et al. 2002). The susceptibility of largemouth bass to infection with this megalocytivirus genotype has been confirmed by field reports of mortalities associated with an ISKNV-like megalocytivirus (Ma et al. 2011). Whilst largemouth bass are currently classified in the family Centrarchidae, recent molecular phylogenetic analysis inferred from a range of nuclear loci has suggested that the centrarchids have a very close affinity with the Percichthyidae (Li et al. 2010), providing additional support that this broader taxonomic grouping may be particularly susceptible to clinical megalocytiviral infection. In terms of marine fish susceptibility, the genus *Oplegnathus,* the monotypic representative of the family Oplegnathidae, is highly susceptible to megalocytiviral infection (Kurita & Nakajima 2012). Although the silver sweep is classified as a

member of the family Kyphosidae (Van Der Laan et al. 2014), mitochondrial gene-based phylogenies have indicated that these 2 families, as well as the Kuhliidae and to a lesser extent the Terapontidae, have a very close affinity with each other (Yagishita et al. 2002, 2009). This provides a situation comparable to the close phylogenetic affinity between the families Centrarchidae and Percichthyidae, both of which contain species that are highly susceptible to megalocytiviral infection. It would therefore be of interest to determine if members of other related families are similarly susceptible to clinical infection with ISKNV-like megalocytiviruses. Given that these families are well represented in Australia and broadly distributed, there is great potential risk for megalocytiviruses to be spread and become established in marine fish in Australia.

The temperature at which the current study was conducted, 28°C, was relatively high, and conducive to clinical expression of megalocytivirus-associated disease and mortality. This temperature is likely to be a seasonal and intermittent occurrence in most areas across the natural distributions of the species used in this study. Although silver sweep is generally considered to be a warm–temperate species, its range does extend to south-eastern Queensland (Edgar 2008), where average sea surface temperatures in late summer and autumn exceed 27°C, and maximum sea surface temperatures can exceed 28°C (www.seatemperature.org/australia-pacific/australia/surfers-paradise.htm). Juvenile silver sweep are frequently found in rock pools (Gomon et al. 2008), which generate micro-environments in which water temperatures can be considerably higher than average sea surface temperatures. For example, Jackson et al. (2013) recorded rock pool temperatures in excess of 28°C in Cape Bank, Botany Bay, although sea surface temperatures for Sydney do not exceed 26°C (www.seatemperature.org/australia-pacific/australia/sydney.htm), and fish have been recorded living in rock pools at a temperature of 41.2°C in the Marshall Islands, even though surrounding maximum sea surface temperatures did not exceed 30°C (Hiatt & Strasburg 1960). Regardless, barred knifejaw was shown to be susceptible to megalocytivirus via direct inoculation over the temperature range of 18–25°C but not susceptible when held at 13°C. Within the susceptible range, at lower temperatures, mean number of days until death was increased compared with treatment groups held at higher temperatures (Jun et al. 2009). Barred knifejaw juveniles that were originally exposed at higher temperatures but then shifted to lower temperatures that were not conducive to clinical expression of disease also survived, a situation mirrored in another study in which fingerling barred knifejaw sourced from a farm which had previously experienced an outbreak of RSIV disease did not express clinical signs of RSIV until shifted to higher temperatures (Choi et al. 2006). Future work could specifically be focussed on the effect of temperature on the megalocytivirus susceptibility of silver sweep.

Similarly, for Murray cod, the freshwater fish model species used in this series of experiments, there are likely to be seasonal periods and local areas in which maximum water temperatures meet or exceed the 27–28°C temperature range used in this series of experiments. This is particularly the case throughout the northern part of the range of this species in the Murray Darling Basin, where maximum surface water temperatures frequently approach or exceed 30°C throughout the summer and early autumn (Hutchison et al. 2011, Ellis et al. 2015). This seasonal period also coincides with the presence of juvenile Murray cod, which are very susceptible to megalocytivirus infection (Lancaster et al. 2003). Likewise, Australian bass juveniles, particularly in the northern part of their range, are likely to be seasonally exposed to maximum water temperatures of 27–28°C, particularly in northern NSW and southeast Queensland, where both riverine and sea surface temperatures approach or exceed this temperature range during late summer and early autumn as outlined above. Again, this period coincides with the presence of juvenile Australian bass, which, as demonstrated in this series of experiments and previously by Go & Whittington (2019), are susceptible to megalocytivirus infection.

Further, as a result of artificial propagation of both Australian bass and Murray cod in hatchery environments, these species may be exposed to sustained high water temperatures in the confines of aquaculture facilities, particularly in late summer and early autumn. This could lead to increased risk for megalocytivirus disease outbreaks, as demonstrated by the epizootic in a recirculating Murray cod facility in Australia during February 2003 (Lancaster et al. 2003).

Although some research has been conducted on the migratory patterns of adult Australian bass (Reinfelds et al. 2013), few data exist in relation to the rate of migration of juvenile Australian bass between marine and freshwater environments. The transition of Australian bass between freshwater and marine environments, and vice versa, over a period of 5 d as used in this study may or may not mimic the natural movements in this species. Regardless, rather than

direct transmission of megalocytivirus from a freshwater fish population to a marine population over the course of several days, as demonstrated in the current study, spread of megalocytiviruses between such fish populations may be possible in a stepwise fashion whereby fish exposed to megalocytivirus in freshwater may travel a short distance downstream before succumbing to the effects of megalocytivirus infection, and in so doing, expose other naïve populations of fish, with this cycle being repeated before transition into marine fish populations.

Other scenarios are also possible. For example, if the temperature-dependent megalocytivirus infection dynamics of barred knifejaw occur in silver sweep and Australian bass, megalocytivirus could disperse from a point source release event to a very wide geographic area because individuals infected by megalocytivirus during warm water conditions in autumn might survive during the cooler winter months until the following spring/summer, by which time the affected individuals may have traversed considerable distances before encountering susceptible populations of naïve fish and succumbing to the effects of megalocytivirus infection. Similarly, there is a potential role in transmission and maintenance of megalocytivirus via species which may have low clinical susceptibility, such as spotted scat *Scatophagus argus* (Chao 2003) or flathead grey mullet *Mugil cephalus* (Gibson-Kueh et al. 2004, Kurita & Nakajima 2012), in a manner analogous to transmission of epizootic haematopoietic necrosis virus (EHNV) by rainbow trout *Oncorhynchus mykiss*, a species that has high morbidity but low mortality to that virus (Whittington et al. 1994, 1996, 1999), although the potential role that such species could play requires further investigation.

Nonetheless, the current study demonstrates an important potential pathway of dissemination of megalocytivirus from freshwater to marine systems. Thus, although Kurita & Nakajima (2012, p. 534) have stated that '[m]egalocytiviruses of freshwater ornamental fish, such as DGIV and ALIV, have almost no opportunity to affect marine fish culture even if these viruses are pathogenic to marine fish species', the current findings suggest that this may not be the case. Consequently, megalocytiviruses from ornamental fish have the potential to pose a much greater biosecurity risk than has currently been accepted. There is the potential for release of megalocytiviruses from ornamental fish culture facilities such as the facility in which ISKNV was detected in ornamental farmed platies *Xiphophorus maculatus* (Rimmer et al. 2015) to surrounding freshwater environments, and, through their capacity to infect euryhaline species, such as Australian bass, to spread megalocytivirus to marine ecosystems and marine aquaculture facilities.

Experimentally, RSIV-like megalocytivirus isolates can infect freshwater fish species (Jeong et al. 2008, Dong et al. 2010), and a natural infection of an RSIV-like megalocytivirus has been reported in freshwater mandarin fish (Fu et al. 2011) and marble goby (Huang et al. 2011, Chen et al. 2013). Given that RSIV has a very broad host range (Matsuoka et al. 1996, Kawakami & Nakajima 2002), it is reasonable to infer that there are likely to be euryhaline fish species with susceptibility to infection with RSIV. Future work could be focussed on testing Australian native species, including euryhaline species, for RSIV susceptibility.

The risks posed by ISKNV-like megalocytiviruses to freshwater native fish species have resulted in a review of biosecurity requirements in relation to the importation of freshwater ornamental fish species into Australia (Biosecurity Australia 2009). This import risk analysis makes reference to the experimental transmission work undertaken by Jeong et al. (2008) on the susceptibility of barred knifejaw to PGIV, an ISKNV-like megalocytivirus, but is restricted to release of ISKNV-like megalocytiviruses into freshwater environments only. In view of the results of this study, the potential for spread to marine environments, and subsequent dispersal, also needs to be considered.

Active stocking programmes are in place for a variety of freshwater fish species in Australia (Gillanders et al. 2006). The scale of these efforts can be considerable, and it has been estimated that over the course of 43 yr, more than 86 million individual fish (comprised of 12 species of native fish and salmonids) have been stocked into NSW waters (NSW DPI 2005). Although such activities can play a significant role in the effective enhancement of freshwater fisheries, they also provide a potential pathway for release of disease agents. For example, in the Shenandoah National Park in Virginia, USA, infectious pancreatic necrosis virus was found in brook trout *Salvelinus fontinalis*, but only in streams with a history of stocking (Panek et al. 2008).

Recently, there has been resurgent interest in Australia for stock enhancement programmes for marine fish species (Loneragan et al. 2013). Such activities could provide pathways for release of megalocytiviruses into marine environments, particularly given that there are hatcheries in Australia that undertake culture of both ornamental and food fish species

(Go & Whittington 2006, Biosecurity Australia 2009). Care is therefore required to minimise the risk of contamination by ensuring that megalocytiviruses are not present in such high-risk facilities, that early detection is possible and notifiable and that eradication be undertaken to prevent establishment and subsequent spread of megalocytiviruses.

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