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ORIGINAL ARTICLE

Effects of freshwater crayfish on influenza A virus persistence in water

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Abstract

Several investigations have recently assessed the ability of some aquatic invertebrates to act as tools for avian influenza A virus (IAV) surveillance as well as their potential role(s) in IAV ecology. Because of this, as well as the high IAV seroprevalence rates noted in select mesocarnivores that commonly inhabit aquatic and semi-aquatic habitats, we evaluated the effects that freshwater crayfish have on IAV in water at three dose levels and monitored for the presence of IAV in crayfish tissues (gill and green gland) and haemolymph at multiple time points. At relatively high, medium and low (approximately 10^4, 10^3 and 10^2 EID_{50}/ml, respectively) doses, mesocosms containing crayfish (*Orconectes* sp.) had less detectable IAV RNA present when final water samples were assayed (9 days post-contact [DPC]). In general, containers without crayfish present had nearly three-fold greater quantities of viral RNA at 9 DPC. A varying number of RNA positive samples were detected for the three crayfish sample types collected. Gill tissue produced the largest number of positive non-water samples (n = 26), with the highest quantities detected from crayfish sampled on 1 and 4 DPC ($10^{3.5}$ EID_{50} equivalent/ml). On a few occasions, gill (n = 8) and haemolymph samples (n = 1) produced higher quantities of viral RNA than their respective water samples or water samples collected 1–2 DPC earlier, but these differences were typically minor. Based upon water samples, statistical models indicated that the interaction of dose and crayfish exposure days explained most of the variation in these data. Future efforts should address if crayfish exposed to IAV-laden water have the capacity to successfully transmit IAVs to mammals and birds which frequently prey upon them.

KEYWORDS

aquatic invertebrate, avian influenza, crayfish, influenza A virus, mesocarnivore, *Orconectes*, persistence, predation, transmission, water

1 | INTRODUCTION

Avian influenza A viruses (IAVs) are often thought to encompass a cycle involving wild birds, such as waterfowl, transmitting the virus through the faecal-oral route within water sources (Brown, Goekjian, Poulson, Valeika, & Stallknecht, 2009). However, certain other wildlife species, such as passerines and peridomestic mammals, are also known to have the capacity to replicate some of these viruses. Recently, multiple workers have evaluated the potential roles of various aquatic invertebrates in the epidemiology of IAVs and as surveillance tools for IAVs (Faust, Stallknecht, Swayne, & Brown, 2009; Huyvaert et al., 2012; Oesterle et al., 2013). During relatively short time periods (e.g. minutes as compared to days in certain other studies), IAV viral RNA concentrations in water flea

(*Daphnia magna*) tissues averaged three times greater as compared to the spiked water in which they were housed; however, no live virus was recovered from the tissues of this aquatic invertebrate (Meixell, Borchardt, & Spencer, 2013). A different study, which was conducted over a lengthier time-frame, indicated that water fleas yielded higher levels of viral RNA as compared to the water in which they were housed at 1, 4 and 6 days after water was spiked with low pathogenic (LP) H4N6 and H5N1 IAVs; however, infectious virus was only detectable in water samples for very brief periods following the addition of virus to water containers with water fleas present (Abbas, Nazir, Stumpf, & Marschang, 2012).

Aquatic invertebrates could impact IAV concentrations in water in multiple ways. For example, the presence of bamboo shrimp (*Atyopsis moluccensis*) was recently reported to be associated with higher concentrations of LP IAV in experimental water containers as compared to containers without shrimp present (Pathak et al., 2018). In contrast, viral titres in water with filter-feeding clams (*Corbicula fluminea*) present were significantly lower than LP IAV-spiked water in the absence of clams (Faust et al., 2009). Further, neither highly pathogenic (HP) IAV-spiked water that was filtered by clams nor clam tissues from clams previously housed in virus-spiked water sources resulted in transmission in wood ducks (*Aix sponsa*) inoculated with these materials (Faust et al., 2009). More recently, the same species of Asiatic clam (*C. fluminea*) was reported to have the capacity to bio-concentrate a LP H6N8 IAV in their tissues, thereby reducing virus concentrations over time in the water where they were housed (Huyvaert et al., 2012). Similar to the avian transmission experiment mentioned above, freshwater snails (*Physa* spp.) exposed to an IAV did not transmit the virus to mallards (*Anas platyrhynchos*) following mallard ingestion of the snails (Oesterle et al., 2013). However, it was reported that zebra mussels (*Dreissena ploymorpha*) have the capacity to accumulate IAVs from spiked water sources and it remains within them for extended periods of time leading the authors to suggest that transmission of IAV to birds predating upon mussels may be possible for long time periods (Stumpf et al., 2010). Clearly, different aquatic invertebrate species, dependent on several factors, have shown variable results in regards to their effects on persistence and/or transience of IAV in water and invertebrate tissues (Table 1).

While laboratory studies investigating IAV in aquatic invertebrates have become commonplace during the last several years, field studies assessing IAVs in aquatic invertebrates are scant. Nonetheless, viral RNA of an IAV was reported in the Mediterranean cone shell (*Conus* sp.) and non-native red swamp crayfish (*Procambarus clarkii*) collected from Mediterranean coastal water and earthen ponds located near migratory bird routes in select Egyptian provinces, respectively (Eissa, Hussein, & Zaki, 2012). Further, IAV RNA in samples (e.g. haemolymph, exoskeleton and organs) collected from red swamp crayfish was recently reported for a second time in Egypt at a prevalence of over 14% (Eissa, Moustafa, El-Desoky, & El-Baky, 2018).

Aquatic invertebrates could potentially have a role in the transmission of IAVs to predatory animals if the virus within the invertebrates' tissues remains infectious (Franklin, VanDalen, & Huyvaert, 2011), as the inactivation of IAVs may be possible in some aquatic invertebrate species (Meixell et al., 2013). An additional consideration is that the aquatic invertebrate in question would need to have a sufficient dose sequestered within it to initiate transmission. Of interest, utilizing a host-pathogen theory framework, a diet composed of aquatic invertebrates was recently suggested as a host trait of birds that may produce a higher susceptibility to pathogens (van Dijk, Verhagen, Wille, & Waldenström, 2018), which may extend to LP IAVs. Thus, if IAV transmission via aquatic invertebrates is possible to animals which prey upon them, animals (both birds and mammals) that commonly utilize and forage within aquatic habitats represent species that are most likely to be associated with this potential transmission mechanism.

Impacts

- The presence of crayfish may reduce the quantity of influenza A virus in water over time.
- Influenza A virus RNA was periodically detected in crayfish tissues.
- Crayfish may not be as useful as an influenza A virus surveillance tool than has been proposed for other aquatic invertebrates.

Some workers have reported high IAV seroprevalence rates in mesocarnivores, such as the raccoon (*Procyon lotor*), in certain locations in the United States and in feral populations in Asia (Hall et al., 2008; Horimoto et al., 2011; Yamaguchi et al., 2014). More recently, IAV genetic material has been detected in feral raccoon samples collected in Japan (Yamaguchi, Fujii, Ogawa, & Imai, 2018). Taken together, these studies suggest that raccoons, which are often associated with aquatic habitats, can be commonly exposed to IAVs in some locations.

Although multiple potential IAV transmission routes to raccoons have been postulated and/or experimentally tested (Hall et al., 2008; Root et al., 2014), the natural mechanisms behind IAV transmission to raccoons are not completely understood. One potential mechanism is aquatic invertebrates. For example, if select aquatic invertebrates have the capacity to bio-concentrate IAVs to levels higher than the water in which they subsist, they could represent a possible mechanism of transmission to animals which prey upon them by helping to bridge the gap at which a dose becomes infectious to a given species. Crayfish can represent a common prey species of raccoons in certain situations (Tevis, 1947).

It is conceivable that crayfish could come into contact with IAVs by multiple routes. First, freshwater crayfish could come into contact with IAVs from the water in which they live if it had been previously contaminated by shedding waterfowl. Second, although less probable, crayfish could come into contact with IAVs while scavenging upon infected bird carcasses. This mode of IAV contact was recently hypothesized for African sharptooth catfish (*Clarias gariepinus*) that fed upon avian carcasses originating from an H5N1-infected poultry farm (Eissa et al., 2012).

TABLE 1 Summary of selected laboratory-based experimental studies associated with influenza A viruses in water with various aquatic invertebrate species present and/or absent

Name	Scientific name	Subtype	Outcome	Reference
Bamboo shrimp	*Atyopsis moluccensis*	H9N2	Higher influenza A virus RNA concentration in water with shrimp present	Pathak et al. (2018)
Water flea	*Daphnia magna*	H3N8	Higher viral RNA loads in tissues as compared to water over short time periods. Negative by cell culture	Meixell et al. (2013)
		H5N1[a]	Higher viral RNA loads in tissues as compared to water over time. Infectious virus not detectable in water fleas or in water at later time points	Abbas et al. (2012)
		H4N6	Higher viral RNA loads in tissues as compared to water over time. Infectious virus not detectable in water fleas or in water at later time points	Abbas et al. (2012)
Freshwater clam	*Corbicula fluminea*	H3N8	Lower viral titres in water with clams present	Faust et al. (2009)
		H5N1[b]	No morbidity/mortality in ducks inoculated with clam tissues or water filtered by clams	Faust et al. (2009)
		H6N8	Viral RNA declined in water and increased in clam tissues	Huyvaert et al. (2012)
Freshwater snail	*Physa* spp.	H3N8	A portion of snail tissues produced live virus but ingestion by ducks did not initiate transmission	Oesterle et al. (2013)
Zebra mussel	*Dreissena ploymorpha*	H5N1[a]	Virus detectable for many days after mussels transferred to freshwater without virus	Stumpf et al. (2010)
Freshwater crayfish	*Orconectes* sp.	H4N6	Lower viral RNA quantities in water with crayfish present	Current study

[a]Low pathogenic.
[b]Highly pathogenic.

The finding of IAV in crayfish in aquatic environments where IAV is endemic (Eissa et al., 2012) is of interest in terms of both the surveillance of IAVs and as a potential transmission mechanism of IAVs to various vertebrate species. Thus, the objectives of this study were to (a) assess the effects of freshwater crayfish on the persistence of an avian IAV in water and (b) assess if select crayfish tissues and haemolymph can concentrate IAV to levels higher than the water in which they are housed. In addition, the information discovered during this experiment is discussed in terms of the potential of crayfish involvement in IAV cycles.

2 | MATERIALS AND METHODS

The potential impact of crayfish on IAV-spiked water was tested by analysing water samples from multiple replicates of water in buckets (hereinafter containers) containing crayfish that were spiked to one of three IAV concentrations and control containers with no crayfish that were spiked to the same levels. Concentrations of IAV were compared in water samples from spiked containers with and without crayfish across multiple days of sampling.

2.1 | Study animals

Wild-caught freshwater crayfish (*Orconectes* sp.) collected in Larimer County, CO, were used in the experiment. A representative sample of individuals from the population where the collection occurred was assayed (see laboratory methods below) prior to the experiment to assess the potential for background IAV in the sampled population. Additional crayfish pairs (n = 33) were used for three virus water treatments and a control group. Each pair was placed in an 11.4 L (e.g. three-gallon) lidded container outfitted with an aerator, a hide (one-half of a piece of 10.2 cm PVC pipe) and feed (algae and shrimp wafers). Life water (municipal water treated to remove chlorine and other chemicals) was used to fill containers. All containers were housed in a BSL-2 animal facility.

2.2 | Influenza A virus water treatments

Overall, forty-two containers were used to house crayfish and/or water in this experiment. Thirty containers were used to house crayfish in water treated with IAV, nine containers were controls treated with IAV (three per dose) but did not have crayfish present in the spiked water, and three were used as control containers that received no virus but had crayfish present to assess the effects of crayfish on evaporation within the containers (Figure 1). The containers were stratified in a 6 × 7 array to abate any spatial bias associated with evaporation rates and room-associated environmental conditions. Initial water volumes were marked on each of the forty-two water containers, and evaporation was assessed from the volume absent at the conclusion of the study. Subsequently, crayfish were exposed to one of three treatments of IAV, which were approximately 10^2 (low dose), 10^3 (medium dose) and 10^4 (high dose) EID_{50}/ml in the containers diluted with

three gallons of life water (see above). The IAV selected, an H4N6 subtype (A/Mallard/CO/P70F1-03/08(H4N6)) (Root et al., 2014), has been commonly detected in wild waterfowl in North America (Piaggio et al., 2012).

Three crayfish, one from each IAV dose treatment group, were sacrificed on each day post-contact (DPC) with IAV-spiked water from 1 to 10 DPC (Figure 1). The three individuals were thoroughly rinsed and then placed in different containers with three gallons of fresh life water and allowed to depurate for approximately 20 min prior to being euthanized. These rinse and depuration procedures were used to help ensure that viral RNA detections were not simply a result of virus on the surface of the sampled crayfish. Following euthanasia, haemolymph, gill and green gland samples were collected from each crayfish. In addition, a 1 ml water sample was taken from each container (n = 42) every DPC from 1 to 10 DPC. Water samples from 1 to 9 DPC were assayed for viral RNA. All remaining crayfish (one per treated bucket with one exception) were euthanized on 11 DPC to collect tissues and haemolymph following a 20 hr depuration period during which the remaining animals of each treatment group were housed in a single container filled with three gallons fresh life water.

2.3 | Laboratory assays

Crayfish tissue samples were extracted as outlined during a pervious study which assayed vertebrate tissues (Shriner et al., 2012). Water, haemolymph and tissue extracts were tested for viral RNA by RT-qPCR following published primers and probes (Spackman et al., 2003) as conducted in earlier experiments (Root et al., 2015). Positive samples were defined as those yielding a two-well positive amplification above a standardized threshold with a C_q value of ≤38.

2.4 | Data analysis

Mixed-effects linear repeated measure regression models were used to analyse these data (after comparing Akaike information criterion (AIC) values for a linear and a mixed-effects model to ensure a mixed-effects model was supported). The data were analysed as a single data set across the three IAV dose levels and as individual data sets representing each dose level. For the full data set, the inclusion of Dose, the number of crayfish exposure days (CFDays) and the CFDays*Dose interaction were evaluated for inclusion by comparing AIC values between the full model and the subset models. These comparisons indicated that the full model was best supported for this analysis. The dependent variable in the model was logDIFF (the logarithm of the viral RNA concentration difference between control and treatment containers), the fixed effects were Dose, CFDays and the interaction between CFDays and Dose, and the random effect was ID (water container identification number). For the models based on individual doses, the dependent variable was logDIFF, the fixed effect was CFDays, and ID was a random effect. The proportion of crayfish remaining within buckets across DPC was accounted for in the CFDays variable, as crayfish were periodically removed from buckets to harvest tissues and haemolymph (see above; this also included one crayfish that was erroneously omitted from bucket 29). Analyses were conducted in R 3.4.3 (R Core Team, 2019).

3 | RESULTS

3.1 | Pre-experiment sampling

Multiple crayfish, which were captured from the same locations as those used in the subsequent experiments, were sampled prior to the initiation of the study to assess if IAV was present in them before experimentation. None of the pre-experiment crayfish samples (i.e. haemolymph, green gland and gill) assayed were assessed to be positive for IAV viral RNA.

3.2 | Control containers

A total of three control crayfish containers (numbers 40–42; Figure 1), which were those that contained crayfish but did not contain IAV, were sampled and assayed from 1 to 9 DPC. None exhibited IAV positive water results for the duration of the experimental period. Further, evaporation levels in these containers at the end of experimental period were similar to the other treatments employed.

3.3 | Influenza A virus water treatments

Evaporation rates were generally low and averaged approximately six-percent of the water volume of all buckets combined. However, no obvious pattern was detected among containers outfitted with crayfish and those without crayfish.

Across all three doses and for both IAV control (IAV present but no crayfish) and crayfish treatment water containers (crayfish and IAV present), virus concentrations in the water declined slowly and consistently across the 9 days of testing. Some trends were noted when water containers associated with the high- and medium-dose treatments with and without crayfish were compared. During early DPC, viral RNA quantities were similar among containers with and without crayfish for both dose treatments. However, during later DPC (e.g. 5–9), higher viral RNA quantities were noted in IAV control containers that excluded crayfish (Figure 2). Statistical model results corroborated these trends with similar results obtained for the full and individual dose data sets. For the full dataset including results for the three dose levels, the interaction between Dose and CFDays was highly significant (p < .001). Based upon individual models for each dose,

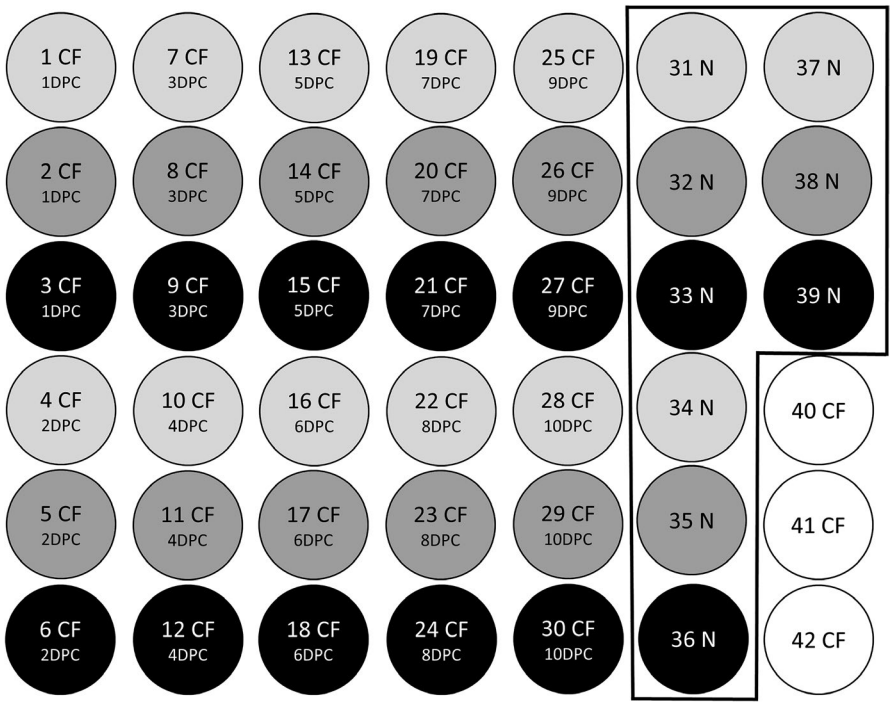

FIGURE 1 Experimental schematic (6 × 7 array) of an influenza A virus study of crayfish and virus-laden water. Each circle represents a water container (numbered 1–42). Containers shown in light grey were inoculated with the low-dose water treatment (approximately 10^2 EID_{50}/ml), those shown in dark grey were inoculated with the medium dose (approximately 10^3 EID_{50}/ml), those shown in black were inoculated with the high dose (approximately 10^4 EID_{50}/ml), and those shown in white did not have influenza A virus (i.e. evaporation control containers). CF indicates the presence of crayfish while N indicates the absence of crayfish (containers outlined in black). A number followed by the letters DPC (e.g. 1DPC) are the day post-contact on which one of the two crayfish were removed from that bucket for tissue testing

the 10^3 treatment approached significance (p = .054) for CFDays and the 10^4 treatment was highly significant (p < .01) for CFDays. Water data associated with the low-dose treatment were less clear (Figure 2). However, although less pronounced/clear, a similar pattern of lower RNA quantities in the water containers with crayfish was noted for this treatment 6–8 DPC (Figure 2). While the CFDays relationship was not statistically significant for the low-dose treatment, this was likely influenced by concentrations of viral RNA falling below the quantitation limit of our assay (e.g. values below log 10 can produce results of zero because viral RNA may not be detected consistently below this level).

3.4 | Gill

Among the three types of crayfish samples tested, gill was the sample type in which IAV viral RNA was most commonly detected (Table 2). Viral RNA was never detected in gill tissue from crayfish

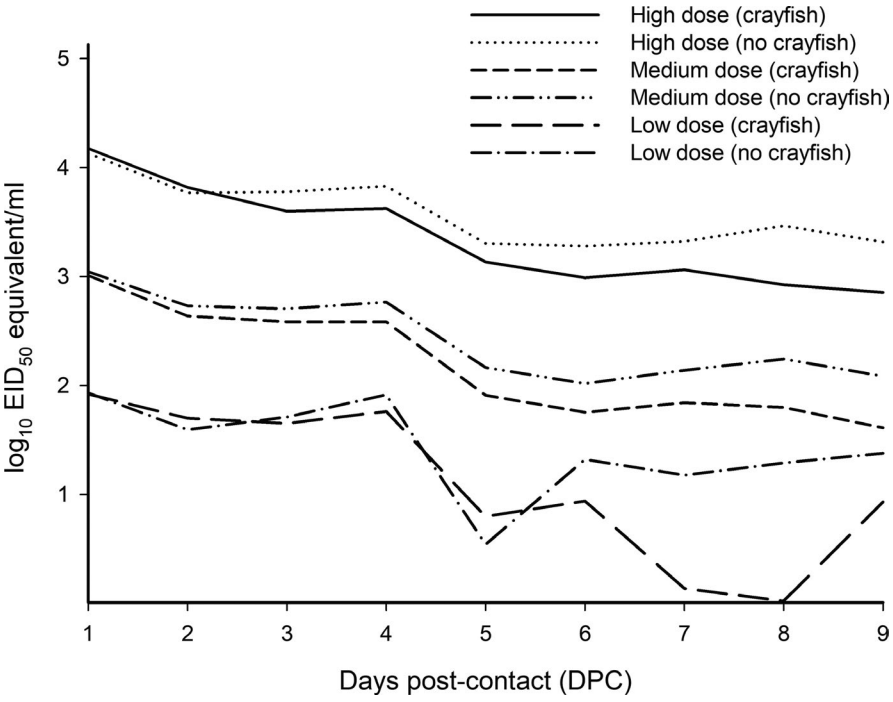

FIGURE 2 Effects of the presence of crayfish on the persistence of influenza A virus in water. Water was spiked to approximately 10^2 EID_{50}/ml (low-dose treatment), 10^3 EID_{50}/ml (medium-dose treatment) and 10^4 EID_{50}/ml (high-dose treatment) in water containers with and without crayfish present

TABLE 2 Viral RNA quantities (calibrated to known EID_{50}/ml samples) of positive freshwater crayfish tissue and haemolymph samples as compared to water samples collected during the same or an earlier time point from the water containers in which the crayfish were housed

Sample type	Dose[a]	Crayfish DPC[b]	Sample quantity[c]	Water quantity[c]	Water day
Gill	M	1	1.41	2.91	1
Gill	M	2	1.76	2.36	2
Gill	M	4	2.27	2.35	4
Gill	M	9	**2.67**	**1.98**	9
Gill	M	10	**1.75**	**1.36**	9[d]
Gill	M	11	**1.31**	**1.01**	9[d]
Gill	M	11	**2.12**	**1.84**	9[d]
Gill	M	11	**1.87**	**0.00**	9[d]
Gill	H	1	3.52	3.88	1
Gill	H	2	3.06	3.66	2
Gill	H	3	2.10	3.46	3
Gill	H	4	**3.53**	**2.92**	4
Gill	H	5	3.02	3.10	5
Gill	H	6	2.19	2.86	6
Gill	H	7	2.54	2.84	7
Gill	H	8	2.11	2.99	8
Gill	H	9	1.35	3.21	9
Gill	H	10	2.68	2.82	9[d]
Gill	H	11	2.64	2.64	9[d]
Gill	H	11	**2.80**	**2.25**	9[d]
Gill	H	11	**3.37**	**2.13**	9[d]
Gill	H	11	1.98	2.77	9[d]
Gill	H	11	2.42	3.21	9[d]
Gill	H	11	1.65	2.79	9[d]
Gill	H	11	1.84	2.82	9[d]
Gill	H	11	2.52	2.67	9[d]
Green gland	H	8	1.86	2.99	8
Green gland	H	10	1.61	2.82	9[d]
Green gland	H	11	1.15	2.58	9[d]
Green gland	H	11	2.31	3.21	9[d]
Haemolymph	H	4	**3.12**	**2.91**	4
Haemolymph	H	5	2.50	3.10	5

Note: Bold numbers represent cases where tissue or haemolymph sample quantities were greater than water quantities.

[a]M = medium-dose treatment (approximately 10^3 EID_{50}/ml); H = high dose treatment (approximately 10^4 EID_{50}/ml).

[b]DPC = days post-contact of crayfish with virus-spiked water.

[c]$\log_{10}$ EID_{50} equivalent/ml.

[d]Water day 9 (i.e. 9 DPC for crayfish) was the last day water samples were assayed.

in low dose water treatment but was detected in multiple individuals associated with the medium and high dose water treatments (n = 26; Table 2). The highest quantities detected were associated with crayfish sampled on 1 and 4 DPC ($10^{3.52}$ and $10^{3.53}$ EID_{50} equivalent/ml), both of which were associated with the high dose water treatment (Table 2). During 11 DPC, when the remaining crayfish were euthanized, the maximum quantity detected was $10^{3.37}$ EID_{50} equivalent/ml, which was an order of magnitude higher in quantity than the water collected from this container during 9 DPC ($10^{2.13}$ EID_{50} equivalent/ml), the last day which water samples were assayed (Table 2). However, the quantity of this individual was much higher than that of the other individual crayfish that were assessed to have positive gill samples during 11 DPC (Table 2). All gill samples tested from control crayfish were negative during 11 DPC.

3.5 | Haemolymph

Only two haemolymph samples were positive for viral RNA (Table 2). These samples were only associated with crayfish in the high dose treatment collected on 4 and 5 DPC, and had a maximum quantity of $10^{3.12}$ EID_{50} equivalent/ml (Table 2). All haemolymph samples tested from control crayfish were negative during 11 DPC.

3.6 | Green gland

Viral RNA in green gland samples was only detected in crayfish from the high dose treatment and was merely detected in four individuals (Table 2). Quantities of viral RNA were low, ranging from $10^{1.15}$ to $10^{2.31}$ EID_{50} equivalent/ml, with the highest quantity produced later in the experimental period during 11 DPC. All green gland samples tested from control animals were negative during 11 DPC.

4 | DISCUSSION

The relatively low levels of IAV RNA detected in the gills of select crayfish may be suggestive of bioaccumulation. However, few gill samples tested had viral RNA concentrations higher than their respective water sample. Of the 26 gill samples that were assessed to be positive for viral RNA, eight exhibited greater viral RNA quantities than the water sample collected from the same container that housed the respective crayfish during the same DPC or during 9 DPC (Table 2). Notably, gill samples collected during 10 and 11 DPC were compared against water samples collected during 9 DPC, the last day water samples were assayed, which suggests that these observed differences may have been even greater if the same days were compared since viral RNA was declining over time. Nonetheless, the relatively small number of gill samples that were of higher quantity than their respective water sample suggests that this observation of apparent bioaccumulation in gill tissue may be more of an exception than a rule. Further, due to logistical and financial constraints, the number of individual crayfish sampled on a given day was limited during this study. A larger sample size may have produced more or proportionally fewer positive tissue samples.

A total of four green gland samples met our definition of positive for viral RNA. The samples were collected on 8, 10 and 11 DPC (Table 2). However, the four positive samples were low in viral RNA quantities, and generally much lower in quantity than their respective water samples (8 DPC) or as compared to those water samples taken at 9 DPC (Table 2), suggesting virus did not bioaccumulate in this tissue.

Only two haemolymph samples met our definition of positive for viral RNA (Table 1). One sample, collected on 4 DPC from a crayfish associated with the high dose water treatment, yielded $10^{3.12}$ EID_{50} equivalent/ml while its respective water sample, also collected during 4 DPC, averaged $10^{2.91}$ EID_{50} equivalent/ml. A second haemolymph sample, which was also associated with the high dose water treatment, averaged $10^{2.50}$ EID_{50} equivalent/ml and its respective water sample averaged $10^{3.10}$ EID_{50} equivalent/ml during 5 DPC (Table 2). The small number of positive haemolymph samples detected in the current study is in contrast to results obtained from field studies of red swamp crayfish in Egypt. For example, RNA positive haemolymph samples were detected in this species from 9 of 16, 4 of 4 and 4 of 4 pooled samples from multiple ponds from three provinces in Egypt (Eissa et al., 2012). The reasons behind the inconsistent haemolymph results of the two studies is unclear; however, the field study did not measure the quantity of virus in the water sources from which red swamp crayfish were collected, and the field virus was likely a different pathotype and was a different subtype than the virus used in the current study.

The presence of bamboo shrimp in water containers spiked with a LP H9N2 IAV was recently reported to be associated with higher viral RNA concentrations in water than containers in which shrimp were absent (Pathak et al., 2018). In contrast, the results of the current study suggest that, although moderate differences were typically detected, the presence of freshwater crayfish in IAV-spiked water containers resulted in lower viral RNA concentration as compared to containers that did not contain crayfish (Figure 2). The reason for this inconsistency is unclear. However, the observation of lower IAV concentrations in water in the presence of aquatic invertebrates in the current study is consistent with other studies (Faust et al., 2009; Huyvaert et al., 2012).

For crayfish to be a viable vehicle of IAV transmission to select terrestrial vertebrates which prey upon them, virus would likely need to be significantly more concentrated within the crayfish as compared to the water in which the crayfish inhabit for successful transmission to occur by this predator-prey mechanism. If not, most vertebrates that may prey upon crayfish (e.g. various birds and mesocarnivores) would more likely receive an infectious dose of virus from repeated ingestion of (birds) or inadvertent nasal inoculation and/or repeated ingestion (mammals) of the water in which the crayfish subsist as compared to consumption of the crayfish. Further, the concentration of IAVs would need to occur without the subsequent inactivation of virus that has been reported for IAVs accumulated by certain other aquatic invertebrate species during previous experiments (Meixell et al., 2013). Of interest, transmission failed in multiple studies that attempted to infect waterfowl with IAV via aquatic invertebrates that were previously housed in water containing various IAVs (Faust et al., 2009; Oesterle et al., 2013). However, a different study documented live virus in zebra mussels 2 weeks after the mussels were placed in freshwater containing no virus, which led the authors to suggest that transmission of virus to predatory water birds may be possible for long periods of time post-accumulation (Stumpf et al., 2010). Thus, experimental transmission studies of mussels, as well as other species such as freshwater crayfish, to various predatory mammalian and avian species would be useful areas of future research to assess the potential role of these aquatic species in IAV epidemiology.

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CONFLICT OF INTEREST

None.

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