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Movement of *Hypophthalmichthys* DNA in the Illinois River Watershed by the Double-Crested Cormorant (*Phalacrocorax auritus*)

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Abstract.—Paired throat and cloacal swabs, along with feather samples, from nesting Double-crested Cormorants (*Phalacrocorax auritus*) at two sites in Illinois, USA, were tested for presence of invasive bighead carp (*Hypophthalmichthys* spp.) DNA. We also used DNA from the feather calamus to determine cormorant sex. Throat and cloacal swabs from cormorants at both locations tested positive for DNA from silver carp (*H. molitrix*), but none tested positive for bighead carp (*H. nobilis*). *Hypophthalmichthys* DNA was not detected on feathers. There were no significant differences among positive *Hypophthalmichthys* DNA detection frequencies between cormorant sexes. To our knowledge, this is the first demonstration of silver carp as part of the Double-crested Cormorant diet in North America. *Hypophthalmichthys* are major invasive species of concern in this region, the detection of water-borne environmental DNA of *Hypophthalmichthys* is an important monitoring tool, and the potential movement of DNA via piscivorous birds may have significant implications for interpreting environmental DNA monitoring data. *Received 8 July 2016, accepted 4 November 2016.*

Key words.—DNA, Double-crested Cormorant, eDNA, feathers, fecal deposition, *Hypophthalmichthys*, *Phalacrocorax auritus*, silver carp, swab samples.

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Invasive bighead carp (*Hypophthalmichthys nobilis*) and silver carp (*H. molitrix*) pose serious ecological and economic threats to aquatic communities in North America (Zhang *et al.* 2016). In the North American Great Lakes Region, invasive *Hypophthalmichthys* are of major concern as populations of both species continue to spread upstream in the Illinois River drainage. Adult *Hypophthalmichthys* have been regularly detected about 65 km southwest of Lake Michigan in the Dresden Cooling Lake, adults and juveniles have recently been observed at Starved Rock Lock and Dam about 130 km from Lake Michigan, but the main invasion front with a large spawning population in the Illinois River is about 180 km away, just north of Peoria, Illinois, USA (U.S. Fish and Wildlife Service 2015). In an effort to prevent the spread of *Hypophthalmichthys* into the Great Lakes, the Electric Dispersal Barrier was constructed about 135 km upstream of the invasion front near Romeoville, Illinois. As part of tracking the spread of *Hypophthalmichthys* in the Illinois River drainage and to verify the effectiveness of the Electric Dispersal Barrier, a large-scale environmental DNA (eDNA) monitoring program has been established to test water samples in the region for *Hypophthalmichthys* eDNA (Jerde *et al.* 2011; Schultz and Lance 2015). Dozens of positive *Hypophthalmichthys* eDNA results have been detected in the Chicago Area Waterway System; however, standard fish community sampling efforts above the Electric Dispersal Barrier have yielded only one adult *H. noblis* and no *H. moltrix* since 2009 (Merkes *et al.* 2014). The potential for *Hypophthalmichthys* DNA to be moved from location to location within the system by piscivorous birds, including the abundant Double-crested Cormorant (*Phalacrocorax auritus*; hereafter, cormorant) is a potentially important factor in interpreting the results from eDNA monitoring (Merkes *et al.* 2014).

To that end, we captured nesting cormorants along the Illinois River and used DNA testing of throat and cloacal swabs, as well as feather samples, to determine if cormorants included *Hypophthalmichthys* in their diets or could transfer carp DNA in their feces or on their feathers.

METHODS

Study Area

From 25-29 May 2012, we captured 15 nesting cormorants at Baker's Lake (42° 08′ 42.3″ N, 88° 07′ 28.5″ W) in Barrington, Illinois. Baker's Lake is a suburban lake managed by the Forest Preserve of Cook County (River Forest, Illinois) and is located about 170 km northeast of the *Hypophthalmichthys* invasion front and about 60 km north of the Electric Dispersal Barrier (Fig. 1). Ongoing monitoring for *Hypophthalmichthys* has found evidence that the invasion front is in the process of moving upstream near the Starved Rock Pool,

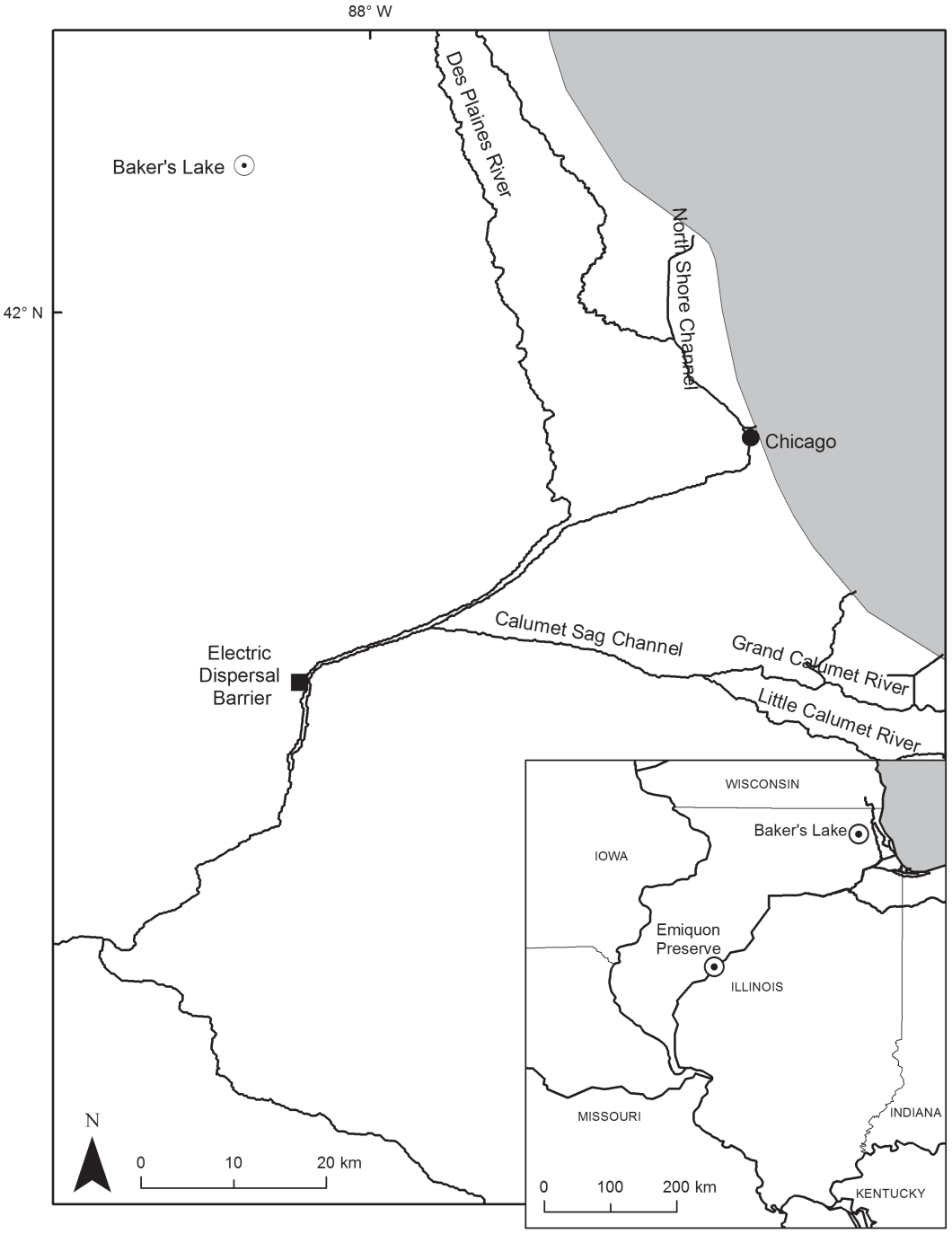

Figure 1. Location of nesting colonies of the Double-crested Cormorant at Baker's Lake and the Emiquon Preserve, Illinois, USA.

about 115 km from Baker's Lake (U.S. Fish and Wildlife Service 2015). Nevertheless, for nesting cormorants at Baker's Lake, *Hypophthalmichthys* are unlikely to be found in nearby water bodies. During the same period, we captured 15 nesting cormorants at The Nature Conservancy's Emiquon Preserve (hereafter, Emiquon Preserve; 40° 21′ 04.3″ N, 90° 05′ 06.6″ W), located near Havana, Illinois. The Emiquon Preserve is managed by The Nature Conservancy and is adjacent to a portion of the Illinois River with an abundant *Hypophthalmichthys* population (U.S. Fish and Wildlife Service 2015). It is about 80 km downstream from the invasive carp front and about 220 km from the Electric Dispersal Barrier (Fig. 1).

Field Methods

We used a small boat to travel to the colony sites where we used modified No. 3 Victor Softcatch padded coil spring traps (King and Tobin 2000) placed on or immediately adjacent to an active nest. After capture (rarely more than 10 or 15 min after trap placement), we freed the cormorant from the trap, placed it in a burlap sack, and used cotton swabs to collect DNA material from the throat and cloaca. We also removed one tail feather and three breast feathers. From these samples, we tested for the presence of *Hypophthalmichthys* DNA. In addition, cormorant DNA in the feather calamus was used to sex the birds. Most captures occurred between 09:00 hr and 18:00 hr. Approximately 30 min was required to process each bird.

Laboratory Methods

We prepared swab samples for extraction by removing the cotton end of the swab with a sterile razor blade and transferring the cotton end into a clean 2.0 ml microcentrifuge tube. DNA was extracted and purified from each sample using the Qiagen's DNEasy Blood and Tissue Kit (Qiagen Inc.) following manufacturer's guidelines with one modification. The volumes of ATL buffer and Proteinase K in the initial lysing step were increased three-fold because of the high absorbency of the swabs. Samples were assayed for the presence of *Hypophthalmichthys* DNA using diagnostic markers and polymerase chain reaction (PCR) protocols described by Jerde *et al.* (2011). All apparent positive results from the PCR assay were subsequently DNA sequenced using forward and reverse primers from Jerde *et al.* (2011), the BigDye Sequencing Kit v3.1 and a Life Technologies 3100 Genetic Analyzer (Life Technologies).

Feathers were stored frozen (-20 °C) in paper envelopes prior to DNA extraction. In testing for *Hypophthalmichthys* DNA, approximately 1 cm of the tip of the feather vane was removed from the largest collected feather. DNA was extracted from the sample using a modified cetyltrimethyl ammonium bromide (CTAB) protocol (Doyle and Doyle 1987). DNA samples were then tested for the presence of *Hypophthalmichthys* DNA using the same PCR protocols described above. For sex determination, approximately 1 cm of the calamus was removed from the base of the largest collected feather. DNA was extracted using the Qiagen DNeasy Blood and Tissue Kit following manufacturer's guidelines. DNA samples were amplified using PCR primers 2550F (5′-GTTACTGATTCGTCTACGAGA-3′) and 2718R (5′-ATTGAAATGATCCAGTGCTTG-3′) and a touchdown thermocycler protocol described by Fridolfsson and Ellegren (1999). PCR reactions consisted of 2.5 µL 10x buffer (including Mg), 0.5 µL each of dNTP solution and primers (10 mM concentration), 0.2 µL of 5PRIME *Taq* polymerase (1 U; 5 Prime, Inc.), 3 µL of DNA extract, and 17.8 µL of water for a total reaction volume of 25 µL. PCR products were separated on 2% agarose gels containing ethidium bromide and visualized under ultraviolet light to identify females (two bands) and males (single band). This procedure was used on 29 of 30 captured cormorants. For one bird, which escaped prior to feather collection, we used cormorant DNA extracted from the throat/cloacal swabs. Tissue samples from four male and two female cormorant specimens of known sex (necroscopic gonadal inspection) were acquired to validate the sex determination technique.

Statistical Analysis

We used a Fischer's Exact Test to determine any differences among sexes in the frequencies of positive *Hypophthalmichthys* DNA found in the cormorant throat and cloacal swabs. All analyses were performed using PROC FREQ (SAS Institute, Inc. 2010).

Results

Of the 15 cormorants captured at Baker's Lake, seven birds (47%) had positive results for *H. molitrix* DNA from the cloacal and/or throat swabs (one bird had positive detections from both cloacal and throat swabs and six had positive results from only throat swabs) (Table 1). Of the 15 cormorants captured at the Emiquon Preserve, 13 (87%) showed positive results for *H. molitrix* DNA in cloacal and/or throat swabs (six birds had positive detections from both cloacal and throat swabs, four had positive results from only cloacal swabs and three had positive results from only throat swabs) (Table 1). No *H. nobilis* DNA was detected in any sample. No *Hypophthalmichthys* DNA was detected on any of the feather samples. Genetic sexing procedures determined that sex ratios were similar at Baker's Lake (seven males, eight females) and at the Emiquon Preserve (nine males, six females) (Table 1). Frequencies of positive *Hypophthalmichthys* DNA detections in the throat and cloacal swabs were not significantly different among sexes ($F = 0.88$, $P > 0.05$).

Table 1. Genetic sexing results (from feathers) and presence of *Hypophthalmichthys* DNA (from throat and cloacal swabs) collected from nesting Double-crested Cormorants captured at Baker's Lake and The Nature Conservancy's Emiquon Preserve, Illinois, USA. eDNA = Environmental DNA.

			Carp eDNA	
Colony	Cormorant ID #	Sex	Throat	Cloaca
Baker's Lake	289	M	Negative	Negative
Baker's Lake	297	F	Positive	Negative
Baker's Lake	295	F	Positive	Negative
Baker's Lake	287	M	Positive	Positive
Baker's Lake	296	F	Negative	Negative
Baker's Lake	285	F	Negative	Negative
Baker's Lake	300	F	Negative	Negative
Baker's Lake	292	M	Negative	Negative
Baker's Lake	286	F	Positive	Negative
Baker's Lake	299	F	Negative	Negative
Baker's Lake	288	M	Positive	Negative
Baker's Lake	291	M	Positive	Negative
Baker's Lake	294	M	Positive	Negative
Baker's Lake	293	F	Negative	Negative
Baker's Lake	290	M	Negative	Negative
Emiquon Preserve	276	F	Positive	Positive
Emiquon Preserve	273	M	Negative	Positive
Emiquon Preserve	275	M	Positive	Positive
Emiquon Preserve	277	M	Positive	Positive
Emiquon Preserve	272	M	Positive	Negative
Emiquon Preserve	271	F	Positive	Positive
Emiquon Preserve	279	M	Negative	Negative
Emiquon Preserve	278	M	Positive	Positive
Emiquon Preserve	274	F	Negative	Negative
Emiquon Preserve	269	M	Positive	Negative
Emiquon Preserve	281	F	Negative	Positive
Emiquon Preserve	270	M	Negative	Positive
Emiquon Preserve	280	F	Positive	Negative
Emiquon Preserve	268	M	Positive	Positive
Emiquon Preserve	282	F	Negative	Positive

DISCUSSION

Hypophthalmichthys DNA was found on both throat and cloacal swabs taken from nesting cormorants, demonstrating for the first time that North American populations of this bird species are predators of the invasive carp. The significant portion of sampled birds from both the Baker's Lake and Emiquon Preserve colonies that tested positive for *H. molitrix* DNA (47% and 87%, respectively) suggests that this fish is a common prey species for cormorants. These results indicate that cormorants have the capacity to move invasive carp DNA into the Chicago Area Waterway System from other regions. We consider the movement of a target species' DNA beyond the known distribution by predators as an "allochthonous eDNA" result to distinguish from other false positive results (e.g., positive results arising from detection of DNA from a non-target species). The detection of *H. molitrix* in the diets of cormorants nesting at Baker's Lake is particularly interesting as there are no known populations near the colony site, and the leading edge of the *Hypophthalmichthys* invasion front is about 115 to 170 km to the southwest. The cormorants at Baker's Lake would have to fly south of the Electric Dispersal Barrier in the Illinois River, or west to the Mississippi River, to forage on smaller-sized *Hypophthalmichthys* (U.S. Fish and Wildlife Service 2015). While cormorants at Baker's Lake do make such

long daily foraging movements during the breeding season, satellite-tagged cormorants were observed to maintain locations north and east of the Electric Dispersal Barrier and largely outside of the known range of *Hypophthalmichthys* (M. P. Guilfoyle, unpubl. data); therefore, the source of carp found in the diets of these cormorants remains unknown.

It is also possible that rather than actively transporting *Hypophthalmichthys* DNA via fecal deposition, cormorants swimming or diving in aquatic habitats where *Hypophthalmichthys* occur may transfer the DNA when fish slime (Merkes *et al.* 2014) or other biological films that contain the carp's DNA adhere to their feathers. However, we could find no evidence of *Hypophthalmichthys* DNA on cormorant feathers. It would also seem unlikely that feathers of other waterbirds, including thousands of ducks and geese in the region, could act to transfer *Hypophthalmichthys* DNA.

More research is needed to comprehensively assess the role of the cormorant and other piscivorous birds as vectors of *Hypophthalmichthys* DNA in the Chicago Area Waterway System. Since it is now known that *Hypophthalmichthys* DNA can be detected in fecal material (Merkes *et al.* 2014), establishing a standardized approach for collecting swab samples from feces-covered nests at large nesting colonies in the Chicago metropolitan area could be a cost-effective approach to determining a timeline for the incorporation of *Hypophthalmichthys* in the diets of nesting cormorants while also assessing the proportion of invasive carp in the diets of cormorants (Symondson 2002; Barrett *et al.* 2007). Understanding the role of highly vagile predators on the movement of DNA and their proportional contributions within a system could improve the utility and power of eDNA monitoring efforts. For example, a better understanding of secondary sources of *Hypophthalmichthys* DNA in the Chicago Area Waterway System, including the role of piscivorous birds, may permit the capability to more effectively model, interpret and distinguish patterns of positive eDNA results arising from secondary sources rather than the actual presence of *Hypophthalmichthys*. Such capability could provide a better assessment tool for determining the current effectiveness of the Electric Dispersal Barrier and other control efforts for preventing *Hypophthalmichthys* from invading the Great Lakes.

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