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PREVALENCE TO *TOXOPLASMA GONDII* AND *SARCOCYSTIS* SPP. IN A REINTRODUCED FISHER (*MARTES PENNANTI*) POPULATION IN PENNSYLVANIA

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ABSTRACT: Understanding the role of disease in population regulation is important to the conservation of wildlife. We evaluated the prevalence of *Toxoplasma gondii* exposure and *Sarcocystis* spp. infection in 46 road-killed and accidentally trapper-killed fisher (*Martes pennanti*) carcasses collected and stored at −20 C by the Pennsylvania Game Commission from February 2002 to October 2008. Blood samples were assayed for *T. gondii* antibodies using the modified agglutination test (MAT, 1:25) and an indirect immunofluorescent antibody test (IFAT, 1:128). For genetic analysis, DNA samples were extracted from thoracic and pelvic limb skeletal muscle from each carcass to test for *Sarcocystis* spp. using 18s-rRNA PCR primers. Antibodies to *T. gondii* were found in 100% (38 of 38) of the fishers tested by MAT and in 71% (32 of 45) of the fishers tested by IFAT. PCR analysis revealed that 83% (38 of 46) of the fishers were positive for *Sarcocystis* spp. Sequence analysis of 7 randomly chosen amplicons revealed the fisher sarcocysts had a 98.3% to 99.1% identity to several avian *Sarcocystis* spp. sequences in GenBank. Data from our study suggest that a high percentage of fishers in Pennsylvania have been exposed to *T. gondii* and are infected with *Sarcocystis* spp.

The fisher (*Martes pennanti*) once inhabited forested regions throughout Pennsylvania (Genoways and Brenner, 1985). The species was extirpated from the state by the early 1900s due to wide-scale deforestation and over-harvesting (Serfass and Dzialak, 2010). The regeneration of over 6.5 million ha of forest habitat in Pennsylvania, combined with public interest, provided the impetus to reestablish a fisher population in the state. During 1994–1998, 190 fishers were translocated from New York and New Hampshire and released at several sites across northern Pennsylvania (Serfass and Dzialak, 2010). Additionally, a fisher population reintroduced to West Virginia in 1969 has expanded into southwestern and south-central Pennsylvania (Ellington, 2010). The fisher is currently designated as a vulnerable species in Pennsylvania and is considered sensitive in U.S. Fish and Wildlife Service Region 5 (Dzialak and Serfass, 2009).

Understanding the role of disease in population regulation is important to the conservation of wildlife (Klyza, 2001). Monitoring the role that various diseases play in wildlife reintroductions is imperative for effectively evaluating disease-associated risks to both reintroduced and resident wildlife (Viggers et al., 1993; Mathews et al., 2006). Furthermore, post-release evaluation of wildlife-disease relations can provide insights to factors that may limit reintroduction success (Larkin et al., 2003). The low population density typical of fishers may reduce the susceptibility of this species to many host-specific, density-dependent diseases (Wobesor, 2007), but their opportunistic feeding habits could increase the probability of exposure to generalist pathogens or parasites, or even to infected sympatric species (Kelly, 1977; Munson and Karesh, 2002; McAllister, 2005). Even those diseases that do not cause massive mortality events can significantly affect a population's fitness due to co-occurring stressors such as habitat fragmentation, encroachment of human development, and any number of human-generated disturbances (Deem et al., 2001), conditions that persist throughout Pennsylvania (Brittingham and Goodrich, 2010).

Toxoplasma gondii and *Sarcocystis* spp. are obligate intracellular protozoans with life cycles that incorporate many stages, i.e., schizonts, meronts, and sarcocysts, within intermediate or definitive hosts (Dubey, Lindsay et al., 2001). Felids are definitive hosts for *T. gondii* and carnivore–scavenger species are definitive hosts for *Sarcocystis* spp. We evaluated the prevalence of *T. gondii* and *Sarcocystis* spp. Both of these parasites have been associated with clinical disease in wild furbearers (Dubey and Lin, 1994; Gerhold et al., 2005), and a thorough review of *T. gondii and Sarcocystis* spp. in wildlife has been published (Dubey and Odening, 2001). However, the epizootiology of these parasites on fisher populations is not fully understood. The seroprevalence of these parasites within fisher populations throughout their range has varied. Forty-five of 77 (58%) fishers from the Hoopa Valley Indian Reservation in California were seropositive for *T. gondii*, with a greater proportion of females exposed (69%) compared to males (29%); however, no differences in exposure were found among age classes (M. Gabriel, pers. comm.). Other records of *T. gondii* infections in fishers include 18% of 28 individuals tested in British Columbia (Philippa et al., 2004), 41% of 379 individuals tested in Ontario (Tizard et al., 1978), and 100% of 2 fishers in captivity (Sedlak and Bartova, 2006).

Little is known of *Sarcocystis* spp. infections in fishers. Clinical meningoencephalitis associated with *Sarcocystis neurona* schizonts was reported in a free-ranging fisher from Maryland (Gerhold et al., 2005). In addition, the fisher had numerous muscle sarcocysts. Although the authors were unable to amplify *S. neurona* DNA from the described muscle sarcocysts at the time of the report, subsequent PCR and sequence analysis attempts from the muscle tissue revealed a 99% identity to *S. neurona* when compared to GenBank accession U07812 (shown in Fig. 1 as UGA internal accession #151-02 *Martes pennanti* muscle sarcocysts). Herein, we report on the seroprevalence of *T. gondii* and *Sarcocystis* spp. infections among free-ranging fishers reintroduced to Pennsylvania.

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MATERIALS AND METHODS

Naturally infected fishers

We obtained 46 road-killed and accidentally trapper-killed fisher carcasses that were collected and stored at −20 C by the Pennsylvania Game Commission from February 2002 to October 2008. With few exceptions, each carcass had an accompanying mortality report that included the date and location of kill, suspected cause of mortality, the name of the collector and, when available, other demographic details regarding the individual animal. From each fisher carcass, an 80-cm^3 sample of quadriceps muscle, a 60-cm^3 sample of forearm muscle, 2 blood samples on Nobuto test strips (Toyo Roshi Kaisha, Ltd., distributed by Advantec MFS, Inc., Dublin, California), and approximately 1–2 ml of clotted blood from the right ventricle were collected.

Pathogen testing

Toxoplasma gondii: For determining the presence of *T. gondii* antibodies, the 2 Nobuto blood-collecting strips were sent to the Animal Parasitic Diseases Laboratory, United States Department of Agriculture, Beltsville, Maryland for analysis. Samples were assayed using the modified agglutination test (MAT) as described by Dubey and Desmonts (1987). The MAT is considered a highly sensitive and specific test (Dubey, 2009). Fisher serum samples were diluted with the serum-diluting buffer in 2-fold dilutions from 1:25 to 1:3200. Serum from an experimentally infected pig was used as a positive control in each plate. Samples with a titer ≥1:25 were considered positive.

Testing for antibodies toward *T. gondii* was also conducted at the University of California, Davis, California using an indirect immunofluorescent antibody test (IFAT) with commercially available slides (VMRD, Pullman, Washington) using cardiac blood samples. The IFAT protocol was slightly modified from previously described methods on a related mustelid, i.e., a sea otter (*Enhydra lutris*) (Miller et al., 2002). Samples were diluted in 1× phosphate-buffered saline solution (PBS) by doubling the dilutions, starting at 1:64 to 1:2,048. Each well had 25 µl of representative diluted sample and the slide was incubated at 37 C for 30 min. After first incubation, slides were washed 3 times in PBS. A 1:100 dilution of fluorescein isothiocynate (FITC)-conjugated goat anti-ferret IgG (Bethyl Laboratories, Montgomery, Texas) was added to each well and slides were incubated at 37 C for 30 min. Slides were washed 3 more times in PBS and mounted with buffered glycerol. Final titers were noted when the complete tachyzoite outline fluorescence was last seen with no apical or peripheral fluorescence. Positive and negative controls from VMRD were used on each slide to demonstrate fluorescence. Positive IFAT cutoffs for *T. gondii* have not been determined for fishers experimentally, but a report of *T. gondii* exposure in fishers detected IFAT titers of 1:320 to 1:640 (Sedlak and Bartova, 2006). We report positive cut-off values of 1:128 and 1:256 as liberal and conservative seroprevalence estimates, respectively.

Sarcocystis ssp.*:* Quadriceps and forearm muscle samples were sent to the University of Georgia Southeastern Cooperative Wildlife Disease Study, Athens, Georgia for *Sarcocystis* spp. testing. DNA samples were extracted from 0.5 g each of thoracic limb and pelvic limb skeletal muscle using a DNeasy Blood and Tissue Maxi Kit (Qiagen, Valencia, California) as per the manufacturer's instructions. DNA amplification of the *Sarcocystis* spp. 18S small subunit rRNA regions was performed using primers 18S9L (5′-GGATAACCTGGTAATTCTATG-3′) and 18S1H (5′-GGCAAATGCTTTCGCAGTAG-3′) (Li et al., 2002). PCR components included 5 µl of DNA in a 25-µl reaction containing 10 mM Tris-Cl (pH 9.0), 50 mM KCl, 0.01% Triton X-100, 2.5 mM $MgCl_2$, 0.1 mM of each dNTP (Promega, Madison, Wisconsin), 1.25 U *Taq* DNA polymerase (Promega), and 0.8 µM of primers 18S9L and 18S1H. Cycling parameters for the amplification were 97 C for 3 min followed by 40 cycles of 94 C for 40 sec, 56 C for 1 min, and 72 C for 1 min 20 sec and a final extension at 72 C for 5 min. For all DNA extractions, a negative water control was included to detect contamination in all PCR reactions. PCR amplicons were separated by gel electrophoreses using a 1% agarose gel, stained with ethidium bromide, and visualized with UV light. An approximate 900-bp amplicon was excised and the DNA purified using a QIAquick® Gel Extraction kit (Qiagen) per the manufacturer's instructions. Seven randomly chosen extracted DNA samples were sequenced using primers 18S9L and 18S1H at the Integrated Biotechnology Laboratories at the University of Georgia using an Applied Biosystems Inc. (Foster City, California) 3100 Genetic Analyzer. Sequences obtained from this study and from other *Sarcocystis* spp. stored in GenBank were aligned using the multisequence alignment Clustal X program (Thompson et al., 1997). Phylogenetic analyses were conducted using the MEGA (Molecular Evolutionary Genetics Analysis) version 4.0 program (Tamura et al., 2007), utilizing the neighbor-joining and minimum evolution algorithms using the Kimura 2-parameter model, and maximum parsimony using a heuristic search. Additionally, 6 randomly chosen muscle samples were fixed in 10% buffered formalin, embedded in paraffin, sectioned at 3 µm, and stained with hematoxylin and eosin (H&E) for light microscopy to examine the morphological features of the *Sarcocystis* sp. muscle cysts.

RESULTS

Thirty-nine mortalities were attributed to vehicular accidents; 4 were accidentally killed by trappers, and the cause of death for 3 fishers was undetermined due to incomplete mortality reports (Table I).

Toxoplasma gondii antibodies were found in 100% (38 of 38) of the fishers tested by MAT (Table I). Samples S59–S66 had inadequate blood on the Nobuto test strips; thus, the results from these samples were not considered reliable. By IFAT, 71% (32 of 45) of the fishers were positive at 1:128 and 40% (18 of 45) were positive at 1:256 (Table I). Collectively, 71% of samples were positive by both tests. Furthermore, many serological samples had high titers (≥512), which may suggest a recent infection with *T. gondii*. Unfortunately, we were unable to verify serological results by bioassay because the carcasses had been frozen long-term, resulting in the death of *T. gondii* (Dubey, 2009).

Using 18S PCR, *Sarcocystis* spp. DNA was found in 83% (38 of 46) of samples (Table I, Fig. 2). Of the positive samples, 32% (12 of 38) were positive from DNA extracted from thoracic limb muscle only, 21% (8 of 38) were positive from DNA extracted from pelvic limb muscle only, and 47% (18 of 38) were positive from DNA extracted from both pelvic and thoracic limb muscle. Nucleotide sequencing revealed a 99.4% identity shared by 3 unique sequences found among the 7 sequenced amplicons in this study. The GenBank accession numbers for the 3 unique sequence groups are listed in Table I. The muscle *Sarcocystis* spp. sequences had from 98.3 to 99.1% identity to a *Sarcocystis* sp. obtained from several avian species including a greater white-fronted goose (*Anser albifrons*) (EU502869), Eurasian sparrowhawk (*Accipiter nisus*) (GU2538884), and rock pigeon (*Columba livia*) (GQ245670). None of the sequences were *S. neurona*. Phylogenetic analysis of the aligned sequences in this study, and other closely related sequences with *Neospora caninum* (NCU17346) as an outgroup, resulted in an alignment 721 bp in length of which 659 were invariant, 22 variable characters were parsimony uninformative, and 40 were parsimony informative. The analysis revealed that the fisher sequences belonged to a separate clade that had robust neighbor-joining (100%), minimum evolution (100%), and maximum parsimony (99%) separation from other *Sarcocystis* spp. (Fig. 1).

Microscopic examination of skeletal muscle sections from PCR-positive samples revealed 0–2 sarcocysts per ×10 field from each examined sample. Measured sarcocyst walls from 10 sarcocysts, from each of 3 fishers with minimal to moderate tissue autolysis, had 1.6 µm-long (range 1–2.5 µm, n = 30) villar protrusions (Fig. 2).

DISCUSSION

Translocations can help restore animals to their native range and can ultimately aid in preventing extinction; however, when doing so, it is important to be proactive about potential disease

TABLE I. Background information and antibody testing results for 46 fishers collected in Pennsylvania from 2002–2008 (NA = not available; NT = not tested).

Animal ID	Sex	Age	Cause of mortality	Date (mo, date, yr)	County of origin	*Toxoplasma gondii* serology (IFAT)	*T. gondii* serology (MAT)	*Sarcocystis* PCR
S1	M	2	Roadkill	2/11/2006	Blair Co.	1:512	1:400	POS
S2	M	1	Roadkill	3/8/2005	Mifflin Co.	1:128	1:50	POS
S3	M	1	Roadkill	2/27/2006	Lycoming Co.	1:64	1:50	POS
S4	F	0	Roadkill	9/23/2004	Unknown	1:128	1:25	POS
S5	M	1	Roadkill	2/15/2002	Lycoming Co.	1:128	1:400	POS
S6	M	8	Roadkill	9/14/2005	Bradford Co.	1:128	1:800	NEG
S7	M	0	Roadkill	Unknown	Somerset Co.	1:512	1:200	POS
S8	M	NT	Roadkill	8/28/2002	Somerset Co.	1:256	1:200	POS
S9	F	2	Roadkill	7/12/2006	Cambria Co.	1:128	1:25	POS
S10	F	2	Roadkill	6/5/2006	Venago Co.	1:256	1:100	POS
S11	M	0	Roadkill	9/19/2006	Warren Co.	1:256	1:100	POS
S12	M	3	Roadkill	1/30/2006	Clarion Co.	1:256	1:400	POS
S13	F	0	Roadkill	1/20/2006	Clarion Co.	1:128	1:50	POS
S14	M	1	Roadkill	Unknown	Clarion Co.	1:256	1:200	POS*
S15	F	0	Roadkill	3/10/2006	Jefferson Co.	1:512	1:>3,200	POS
S16a	M	2	Roadkill	Unknown	S.W. PA	1:512	1:400	NEG
S16b	M	—	Unknown	Unknown	Unknown	1:128	1:>3,200	POS†
S17	M	0	Roadkill	12/18/2002	Westmoreland Co.	1:128	1:400	POS*
S18	M	1	Roadkill	5/13/2005	Fayette Co.	1:128	1:200	POS*
S19	F	0	Roadkill	03/2004	Cambria Co.	1:64	1:100	POS
S48	F	0	Roadkill	05/07/2007	Cambria Co.	1:64	1:200	POS
S50	M	2	Roadkill	3/4/2006	Forest Co.	1:256	1:200	POS
S51	M	2	Roadkill	3/1/2007	Centre Co.	1:256	1:200	POS
S52	F	5	Roadkill	2006–2007	Somerset Co.	1:512	1:800	POS*
S53	F	1	Roadkill	2006–2007	Fayette Co.	1:64	1:50	POS
S54	F	4	Roadkill	06/2007	Cambria Co.	1:128	1:50	POS
S55	F	1	Roadkill	2006–2007	Cambria Co.	1:64	1:200	POS‡
S56	M	7	Roadkill	3/11/2007	Forest Co.	1:128	1:100	NEG
S57	F	1	Roadkill	10/30/2006	Warren Co.	>1:64	1:50	POS
S58	M	0	Roadkill	3/7/2006	Jefferson Co.	>1:64	1:50	POS*
S59	M	NA	Roadkill	10/23/2007	Jefferson Co.	>1:64	NT	POS
S60	F	NA	Trapper-killed	1/22/2008	Somerset Co.	1:256	NT	POS
S61	F	NA	Trapper-killed	12/14/2007	Somerset Co.	1:128	NT	POS
S62	F	NA	Roadkill	2/21/2008	Clarion Co.	1:256	NT	POS
S63	F	NA	Roadkill	2/21/2008	Clarion Co.	1:256	NT	POS
S64	F	NA	Roadkill	3/17/2008	Clarion Co.	>1:64	NT	NEG
S65	F	NA	Roadkill	7/11/2007	Venago Co.	1:256	NT	NEG
S66	M	NA	Trapper-killed	1/14/2008	Mercer Co.	1:256	NT	POS
S68	M	NA	Roadkill	8/31/2008	Union Co.	NT	1:50	POS
S69	M	NA	Roadkill	2/15/2006	Tioga Co.	1:256	1 50	NEG
S70	F	NA	Roadkill	9/22/2006	Elk Co.	1:128	1:25	POS
S71	M	NA	Unknown	5/16/2008	Bedford Co.	1:128	1:400	POS
S72	M	NA	Roadkill	11/17/2006	Luzerne Co.	>1:64	1:50	NEG
S73	M	NA	Trapper-killed	1/18/2008	Columbia Co.	>1:64	1:25	NEG
S74	F	NA	Unknown	Unknown	Unknown	>1:64	1:25	POS
S75	F	NA	Roadkill	10/26/2008	Centre Co.	>1:64	1:25	POS
Total positives						≥1:128 (71%:32 of 45) ≥1:256 (40%:18 of 45)	≥1:25 (100%: 38/38)	(82.6%:38/46)

* Sequence group A and (=) Genbank no. S14 = HQ709139; S17 = HQ709141; S18 = HQ709142; S52 = HQ709137; and S58 = HQ709138.
† Sequence group B and (=) Genbank no. S16b = HQ709140.
‡ Sequence group C and (=) Genbank no. S55 = HQ709143.

risks (Gaydos and Gilardi, 2004; Mathews et al., 2006). This includes monitoring reintroduced populations for diseases and understanding how various diseases could negatively impact a reintroduction effort.

Data from the present study suggest that a high percentage of Pennsylvania fishers have been exposed to both *T. gondii* and *Sarcocystis* spp. The high prevalence of *T. gondii* in Pennsylvania fisher is not surprising given that these are terrestrial predators,

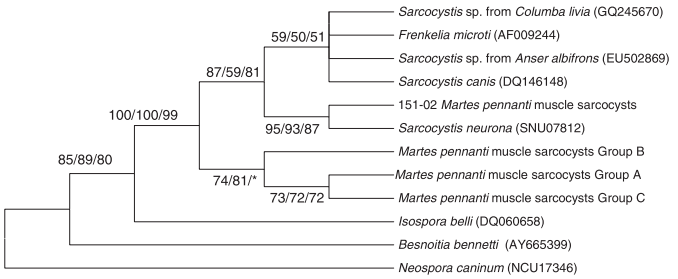

FIGURE 1. Phylogenetic analysis of *Sarcocystis* spp. isolates from this study and other Ampicomplexa based on sequence alignment of overlapping 721 bp 18S rRNA gene. The tree was constructed using a minimum evolution algorithm with 500 replications in a Kimura 2-parameter model with *Neospora caninum* as an outgroup. Unique sequences from *Sarcocystis* spp. sequences obtained in this study are designated by letters; see Table I for individual fisher sequences belonging to each sequence group. Bootstrap values for neighbor-joining/minimum evolution/maximum parsimony values are shown at the nodes. Asterisks indicate nodes with bootstrap values below 50%.

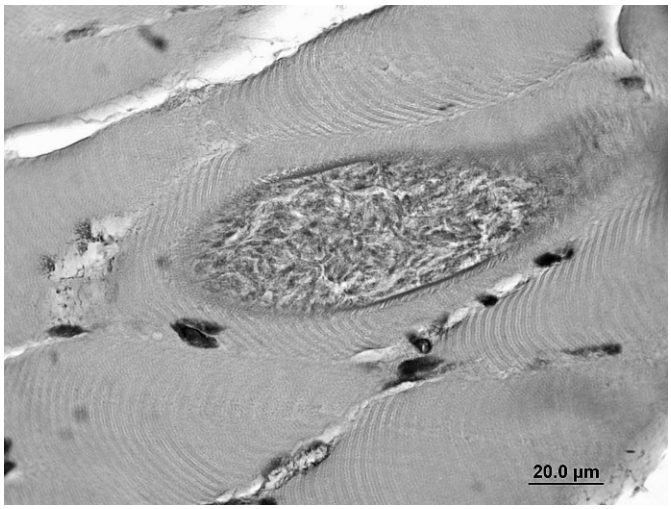

FIGURE 2. Sarcocyst in skeletal muscle of a Pennsylvania fisher. H&E. Bar = 20 µm.

and past studies found *T. gondii* prevalence rates of 10% to 60% in several prey residing in the state, prey that have been detected in Pennsylvania fisher stomachs as well as in other community carnivores (Dubey et al., 1995; Humphreys et al., 1995; Stewart et al., 1995; De Thoisy et al., 2003; Mucker et al., 2006; Wester, 2009). Toxoplasmosis in carnivores is typically subclinical but can develop disseminated disease leading to morbidity or mortality when concurrently infected by an immunosuppressive agent such as canine distemper virus (Diters and Nielsen, 1978; Van Moll et al., 1995; Dubey and Odening, 2001). It should also be noted that, while the seroprevalence for *T. gondii* in fishers was high in our study, infection was not confirmed histologically or by molecular techniques. Future research should incorporate methods that provide seroprevalence values and infection data of *T. gondii* for a better understanding of the epidemiology and ecological role this parasite plays within this host species natural history.

The identification of muscle cysts indicates that fishers are an intermediate host for the *Sarcocystis* spp. The definitive host(s) for these *Sarcocystis* spp. are likely predatory or scavenging vertebrates. Further research, including molecular surveys of sporocyst and experimental infection of potential definitive hosts, would be useful for elucidating the epizootiology of the *Sarcocysts* spp. Additionally, it is unknown whether fishers are only intermediate hosts or are possibly definitive hosts for selected *Sarcocystis* species, or if any of these fishers were infected with *S. neurona* in the central nervous system, as only muscle was available for testing. It is possible that the fisher is an aberrant host that accidentally enters the life cycle of some *Sarcocystis* spp., similar to the horse (*Equus ferus*) and *S. neurona* (Dubey, Saville et al., 2001). However, if this was true, we would not expect the genetic sequences of the sarcocysts from the fishers we sampled to be so similar or to have as high an infection rate as that seen in our study. Phylogenetic analysis of the sequences in the present study gave robust support for separation of the fisher sequences from other *Sarcocystis* spp. used in the analysis, and none of the 8 *Sarcocystis* nucleotide sequences from this study match specifically to *S. neurona* (Fig. 1). In the absence of transmission electron microscopy, we were unable to further characterize the species of *Sarcocystis*.

The high proportion of individuals infected with both *T. gondii* and *Sarcocystis* spp. warrants more research into both the clinical and sub-clinical effects of these protists for fisher populations. For example, black-footed ferrets (*Mustela nigripes*) and raccoons (*Procyon lotor*) infected by morbilliviruses, such as CDV, have been proven more susceptible to *T. gondii* and *S. neurona* infections (Williams et al., 1998; Hancock et al., 2005). As such, the possibility of co-exposures should be considered when examining fishers for diseases. Future research should also investigate whether individuals infected with either *T. gondii*, *Sarcocystis* spp., or both are more susceptible to vehicular strikes than are uninfected animals, as the majority of the animals we sampled died in this manner. In a marine mustelid, the sea otter, it was documented that white shark (*Carcharodon carcharias*) predation was 3.7 times more likely to occur on individuals with moderate to severe *T. gondii* infections (Kreuder et al., 2003). Behavioral changes induced by *T. gondii* or *Sarcocystis* spp. could influence fisher feeding ecology, movement, reproductive success and, ultimately, survival.

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