QUANTIFYING *ICHTHYOPHONUS* PREVALENCE AND LOAD IN PACIFIC HALIBUT
(*HIPPOGLOSSUS STENOLEPIS*) IN COOK INLET, ALASKA

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ABSTRACT

Quantifying *Ichthyophonus* prevalence and load in Pacific halibut (*Hippoglossus stenolepis)* in Cook Inlet, Alaska

By Caitlin Grenier

Ichthyophonus, a non-specific fungus-like protozoan fish parasite, has caused epizootic events among economically important fish stocks including herring and salmon and can result in reduced growth, stamina, and overall fish health. Recently Ichthyophonus was detected in Pacific Halibut (*Hippoglossus stenolepis*) in Cook Inlet, Prince William Sound, Gulf of Alaska, and the Bering Sea. During the summers of 2012 and 2013 I sampled sex, length, age, diet composition, heart, spleen, liver, and kidney tissues from 563 halibut (364 females, 199 males) landed by the Homer sport-charter fishery using the "pre-dumpster, post-mortem" method. *Ichthyophonus* prevalence was determined by MEM culture and parasite load (schizonts/ gram) was determined using pepsin digestion. In 2012, 23% of the fish sampled had *Ichthyophonus*; 29% in 2013. We found no evidence of the parasite in liver, spleen or kidney tissues and there was no difference in prevalence between males and females. Pepsin digestion analysis indicates a wide range of parasite load among infected fish with 6 to 1,245 Ichthyophonus schizonts per gram of heart tissue. Analyses to determine the diet composition of sick fish using gut contents and stable isotopes are ongoing.

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GENERAL INTRODUCTION

An increase in marine epidemics, both in number and frequency, has recently been acknowledged (Harvell et al. 1999; Sherman 2000). This increase, particularly within the past 30 years, presents scientists and policy makers with opportunities to explore potential adverse impacts on ecological and economic systems. One pathogen that causes recurring epizootics among many commercially important marine and freshwater fishes, resulting in negative economic impacts, is *Ichthyophonus hoferi* (Kramer-Schadt *et al*. 2010; Kocan et al 2010; McVicar 2011). This fungus-like protistan parasite was first noted in rainbow trout in 1893 by German scientist von Hofer, has since been documented in over 100 fish species, and has significantly impacted populations of Atlantic and Pacific herring (*Clupea pallasi*), mackerel (*S. scombrus*), yellowtail flounder (*Pleuronectes ferruginea*), Chinook salmon (*Oncorhynchus tshawytscha*), and American shad (*Alosa sapidissima*) (Burge *et al.* 2014; McVicar 2011).

External signs of *Ichthyophonus* infection in some hosts include skin roughening (the "sand paper" effect), black papules, ulceration (particularly in the lateral muscle), and wasting of musculature (Kocan *et al*, 2004, McVicar 2011). The severity of gross clinical signs depends on the fish species and is an indication of infection intensity. Generally, the parasite affects multiple internal organs; primarily those well supplied with blood (McVicar, 1984). Organs and tissues with heavy infections often present with white nodules, consisting largely of multinucleated schizont(s) and a surrounding granulomatous host response (Kocan *et al.*, 2006). Different host species and even individuals of the same species show a range of responses to infection (McVicar and McLay, 1985). For example, Pacific herring are highly susceptible to infection and frequently display external signs; however species such as flounder and mackerel typically do not show any external signs (McVicar, 2011). Primary infection sites also vary

among species. In Pacific herring the heart is the most affected organ, which also holds true for salmonids (Jones and Dawe, 2002; Kocan *et al.*, 2011). In rockfish, however, it is the liver, kidney, and spleen (Kent *et al*. 2001). Possible reasons for these differences include varying host physiology, cellular reaction to infection, and different *Ichthyophonus* species/strains (Kocan *et al*. 2006).

Piscivorous fish become infected by consuming infected prey (Kocan *et al.* 2010). Spanggaard and Huss (1996) determined that infectious cells can be released from the gills, intestinal mucosa, kidney tubules, and necrotic areas of the skin; further, schizonts isolated from marine species can remain viable in seawater for almost two years. Once in the host, parasite invasion and dissemination occurs quickly, and is detectable at 6 hours post exposure in aortic blood cultures within experimentally infected Pacific herring and sculpin (Kocan *et al*., 2013). In another study examining *Ichthyophonus* dissemination and pathogenicity, experimentally infected herring died as soon as 7 days post exposure, with no external signs, while other herring in the same experiment survived and began to display external clinical signs at 36 days post exposure (Kocan *et al.* 1999). Hershberger *et al.* (2002) determined that the prevalence of infection increases with age of wild Pacific herring, presumably because the probability of exposure to the parasite increases with age. McVicar (2011) suggested that once a fish becomes infected the fish never recovers. The infection either causes acute mortality of the host or enters a chronic phase.

Alaska is home to some of the most productive and lucrative commercial fisheries in the world. Fish is Alaska's number one export by weight and is second, only to oil, in producing total revenue for the state, bringing in approximately $4.6 billion dollars annually (ADFG, 2014). Pacific halibut (*Hippoglossus stenolepis)* are the most economically important groundfish

species in Alaska and have been managed by the International Pacific Halibut Commission (IPHC) since 1923. The IPHC is charged with managing halibut populations by assessing the stocks and determining catch limits to obtain the maximum sustainable yield. Other partners that manage Pacific halibut in Southcentral Alaska include the National Marine Fisheries Service (NMFS) and the Alaska Department of Fish and Game (ADF&G). The NMFS is responsible for developing, implementing, and enforcing regulations, suggested by the IPHC, for Pacific halibut management in U.S. waters. The ADF&G helps regulate at the state level by holding a seat on the North Pacific Fishery Management Council (NPFMC), which issues licenses to sport fishermen and guides, as well as monitoring the sport and subsistence fisheries.

In 2011, 14,670M lbs. of halibut were landed by commercial fishermen in fishing area 3A (including Southcentral Alaska ranges from Cape Spencer to Kodiak Island), with 41% of that total caught in the Cook Inlet (IPHC 2014; Figure 1). The total catch by commercial fisherman in Alaskan waters was estimated at 36 M lbs. pounds in 2011 (IPHC 2014). However due to a recently acknowledged decrease in size at age among halibut, the coast wide catch limit was reduced in 2013, from the 2012 limit of 33.5 M lbs., to 31 million pounds (IPHC 2014). The mean weight / halibut also decreased in 2012 to 12.7 lbs., down from 14.7, representing the lowest estimated average weight since ADF&G began monitoring charter harvests in the 1990's (ADF&G, 2014). Several untested hypotheses exist to account for the observed decreased size at age, including diet and parasitism (IPHC 2013). Both diet and parasitism can have profound effects on physiology, such as reduced growth and decreased organ function. Considering that parasites can be transferred to hosts through diet, these two components are becoming increasingly important to study.
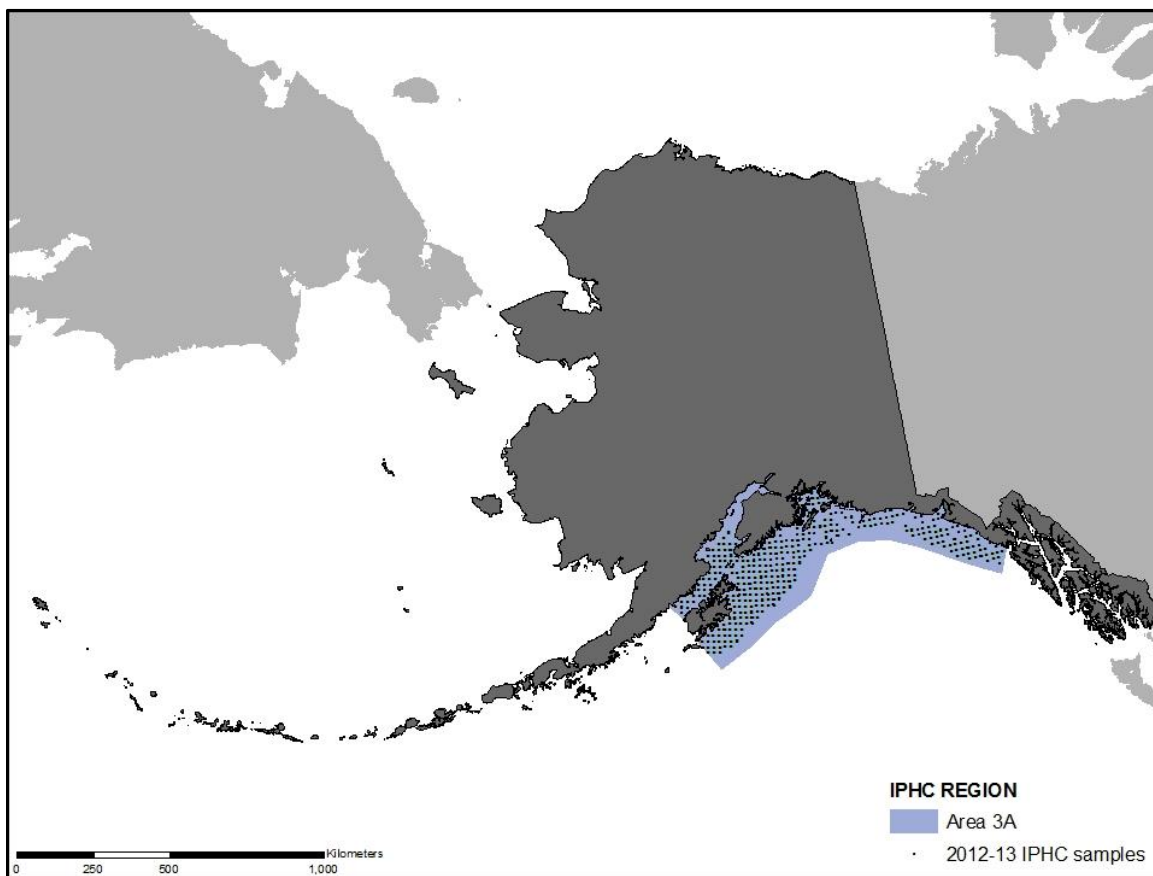
Figure 1 International Pacific Halibut Commission regulatory area 3A; points are all of the sample locations for the 2014 annual stock assessment survey (IPHC 2014).

In 2011, Alaska Pacific University students enrolled in an Ichthyology class sampled marine fishes for *Ichthyophonus hoferi* at five ports in Alaska: Whittier, Valdez, Seward, Homer, and Ninilchik. As a result of this sampling, *Ichthyophonus* was detected in five new marine hosts, including Pacific halibut (*Hippoglossus stenolepis)*, black rockfish (*Sebastes melanops),* yellow-eyed rockfish (*Sebastes ruberrimus)*, lingcod (*Ophiodon elongatus*), and Pacific cod (*Gadus macrocephalus)*. Among the five ports, Pacific halibut were the most sampled fish and had the highest overall prevalence (n = 377; 32.6% prevalence).

Due to the lack of baseline knowledge of *Ichthyophonus* in Alaskan waters, the North Pacific Research Board (NPRB) has funded an ongoing monitoring program. Recently, *Ichthyophonus* infection was detected in a preliminary study in Pacific halibut ranging from Cook Inlet to the Bering Sea, Alaska (NPRB, semiannual report, project #1015, 2013). However, basic epizootiological questions remain unaddressed, including the geographic range, infection prevalence and intensity, seasonality, and virulence of *Ichthyophonus* to Pacific halibut. The objectives of this study were:

1) To determine infection prevalence and how factors, such as sex, length, and age affect the prevalence of *Ichthyophonus* in Pacific halibut within Cook Inlet, Alaska;

2) To develop a new method of *Ichthyophonus* detection, using pepsin digestion, that is capable of quantifying parasite load.

CHAPTER 1

PREVALENCE OF *ICHTHYOPHONUS* IN PACIFIC HALIBUT (*HIPPOGLOSSUS STENOLEPIS*) IN COOK INLET, ALASKA

1.1 INTRODUCTION

In 2011, *Ichthyophonus hoferi*, a cosmopolitan pathogenic parasite, was detected in Pacific halibut (*Hippoglossus stenolepis)* in Cook Inlet, Alaska. Epizootics of *Ichthyophonus* have caused significant ecological and associated economic losses among commercially important fish stocks such as Atlantic and Pacific herring (*Clupea pallasi)*, Yukon River Chinook salmon (*Oncorhynchus tshawytscha*), and rainbow trout (*Oncorhynchus mykiss*) (Kocan *et al.* 2009). First noted in brown trout (*Salmo trutta*) in 1893 by German scientist von Hofer, it is known to infect more than 100 marine and freshwater fish species worldwide (Hershberger *et al.* 2002). Generally, the parasite affects multiple internal organs; primarily those highly vascularized (McVicar 2011). Clinical signs of *Ichthyophonus* infection can include exterior ulcers, wasting of musculature, and white nodules throughout soft tissues and infected organs. Experimentally infected fishes, such as rainbow trout and Chinook salmon, exhibit decreased swimming stamina, reduced growth rates, increased post-exertion recovery periods, and overall reduced health (Kocan *et al.* 2010).

Piscivorous fishes become infected with *Ichthyophonus* by consuming infected prey (Kocan *et al.* 2010). It is unclear how planktivorous fish acquire the disease, but Hershberger *et* al. (2002) speculated that copepods are potential paratenic or transfer hosts. Different species, and even individuals of the same species, show a range of tolerance or resistance to infection (McVicar and McLay, 1985). For example, Pacific herring are highly susceptible to infection and frequently display gross signs, while fish such as flounder and mackerel do not show any external signs (Sindermann and Scattergood, 1954). Once in the host, parasite invasion and dissemination occurs quickly. Experimentally infected herring died as soon as 7 days post

exposure, with no external signs, while other herring in the same experiment survived and began to display external clinical signs at 36 days post exposure (Kocan *et al.* 1999).

Hershberger *et al.* (2002) determined that in both experimentally and naturally infected Pacific herring, prevalence increases with age, possibly due to recurrent interactions with *Ichthyophonus*, with other cohorts often a source of infection for juveniles. McVicar (2011) suggested that once a fish becomes infected the fish never recovers. The infection either causes acute mortality of the host or enters a chronic phase.

In 2011, 14.67 M lbs. of halibut were landed by commercial fishermen in fishing area 3A, with 41% of that caught in the Cook Inlet (IPHC 2012). Fishing area 3A in Southcentral Alaska ranges from Cape Spencer to Kodiak Island (Figure 1). The total catch by commercial fisherman in Alaskan waters was estimated at 36 M lbs. in 2011 (IPHC 2013). However, due to a recently acknowledged decrease in size at age among halibut, the coast wide catch limit was reduced from the 2012 limit of 33.5 M lbs. to 31 M lbs. (IPHC 2013). It is estimated that the average mean weight decreased in 2012 to 12.7 lbs., from 14.7, and is the lowest estimated average weight since the Alaska Department of Fish and Game (ADF&G) began monitoring charter harvests in the 1990's (ADF&G 2012). Several untested hypotheses exist to account for the observed decreased size at age among halibut, including diet and parasitism (IPHC 2013). Both diet and parasitism can have profound effects on physiology such as parasitic castration, reduced growth, and decreased organ function (IPHC 2013, Marcogliese 2005). Considering parasites can be transferred to hosts through diet, these two components are important to study. Parasites are ubiquitous in all ecosystems, and in turn can be used as health indicators. Due to intricate life cycles, both inside and outside of their host, parasites are typically more sensitive to

environmental changes, therefore allowing them to be an initial indicator of ecosystem change (Marcogliese 2005).

In 2011, Alaska Pacific University students enrolled in an Ichthyology class sampled for *Ichthyophonus hoferi* at five ports in Alaska: Whittier, Valdez, Seward, Homer, and Ninilchik. In addition to assessing the prevalence of infection in all species of sport-caught fish, this sampling was intended to test for a relationship between *Ichthyophonus* infection and "mushy halibut syndrome (MHS)," a condition resulting in flaccid or opaque musculature associated with severe muscle fiber atrophy and necrosis in Pacific halibut (IPHC 2014). This study found no relationship between *Ichthyophonus* infection and MHS (APU – FAST Lab unpublished data). As a result of this sampling, five species without previous documentation of *Ichthyophonus* infection were found: Pacific halibut (*Hippoglossus stenolepis)*, black rockfish (*Sebastes melanops),* yellow-eyed rockfish (*Sebastes ruberrimus)*, lingcod (*Ophiodon elongatus*), and Pacific cod (*Gadus macrocephalus)*. Among the five ports, Pacific halibut were the most sampled fish and had the highest overall prevalence (N = 377; 32.6% prevalence).

Due to the lack of baseline knowledge of this parasite in Alaskan waters, the North Pacific Research Board (NPRB) has funded ongoing monitoring programs. Recently, *Ichthyophonus* infection was detected in a preliminary study in Pacific halibut ranging from Cook Inlet to the Bering Sea, Alaska (NPRB, semiannual report, project #1015, 2013). However, basic epizootiological questions remain unaddressed in Pacific halibut including the parasite's geographic range, infection prevalence and intensity, seasonality, and virulence. The objectives of this study were to determine *Ichthyophonus* prevalence and how factors, such as sex, length, and age affect prevalence in Pacific halibut within Cook Inlet, Alaska, through the use of sport caught fish sampled in the port of Homer. This is the first effort to detect and quantify

Ichthyophonus infection using a protocol designed around sport fishery port sampling in any fish species.

1.2 METHODS AND MATERIALS

Sampling

Halibut were sampled in the port of Homer in collaboration with the Alaska Department of Fish and Game port-sampling program. Located in lower Cook Inlet, Homer supports the largest Pacific halibut sport fishery in the United States (Meyer and Powers 2013). Most of the local fishers and charter fishing operations fillet their catch at fish processing stations located at the port. At these stations ADFG staff interview fishers and sample the catch. Once filleted, the carcasses are discarded in dumpsters, ultimately ground and dumped at sea. Samples were collected from carcasses, prior to their disposal in the dumpsters; biological data from each fish included fork length (cm), sex (2012 Field Procedure Manual for the Southcentral Alaska Halibut and Groundfish Harvest Assessment Program of ADF&G), and age (determined from sagittal otoliths). Our aim was to sample at least 30 male and 30 female halibut in as many 10 cm fork length size bins as possible (< 39 cm, $40 - 49$ cm, $50 - 59$ cm, etc.)

Prevalence of *Ichthyophonus* was determined by tissue explant culture. Heart, kidney, liver, and spleen samples, approximately 0.5 gram, were collected using aseptic technique, visually examined for signs of infection (white nodules, ulcers etc.) and placed in tubes containing Eagles Minimum Essential Medium (supplemented with 5% fetal bovine serum, glutamine, gentamicin, penicillin, streptomycin, and buffered to pH 7.5 with HEPES MEM; Hershberger *et al.* 2002). In the spring of 2012 I was trained, for one week at the USGS Marrowstone Marine Field Station, in detecting *Ichthyophonus* schizonts and germinating bodies in tissue explant cultures. To

determine infection, cultures were placed in a Percival incubator at 15 °C and inspected after one and two weeks using a Zeiss inverted microscope for the presence of *Ichthyophonus* schizonts and or germinating bodies. During 2012, only hearts cultured positive. No *Ichthyophonus* stages were detected in any cultured liver, kidney, or spleen. Therefore, explant cultures in 2013 contained only heart tissues.

Analysis

Ichthyophonus prevalence (100 X number of infected fish divided by the total number of fish sampled) was calculated overall and by sex. Proportional differences were examined with χ^2 tests to determine whether *Ichthyophonus* prevalence differed between males and females and between sampling months. To determine whether fish with *Ichthyophonus* exhibited slower growth we examined the differences in mean size at age for all ages in both genders for infected and uninfected halibut. Mean age at size was also examined. Finally, I examined the relationship between *Ichthyophonus* infection and age with linear regressions.

1.3 Results

The combined prevalence of *Ichthyophonus* in Pacific halibut from Lower Cook Inlet was 26% (146 / 563) during 2012 and 2013. Prevalence increased from 22.6% (71 / 315) in 2012 to 29.5% (73/248) in 2013, but this difference was not significant (P = 0.125, χ^2). Among 40 fish where multiple organs (heart, liver kidney, and spleen) were sampled, the parasite was detected in only heart tissue (n = 16).

Month variability

There was no difference in infection prevalence between the sampling months of June, July, and August during either 2012 (P = 0.50, χ^2) or 2013 (P = 0.11, χ^2)

Sex

Infection prevalence was higher in females (25%) than in males (22%); however the comparisons were not significant (P = 0.26, χ^2; Table 1.1, Figure 1.1). The youngest sampled female to culture positive for *Ichthyophonus* infection was 3 yrs., with the oldest being 22 yrs. (Figure 1.2). The youngest sampled male to culture positive for infection was 6 yrs., with the oldest being 29 yrs. (Figure 1.3).

Table 1.1 Combined *Ichthyophonus* prevalence by sex for 2012 and 2013

Sex	No Ich	Ich	Prevalence
Female	272	92	0.25
Male	156	43	0.22
Combined	428	135	0.24
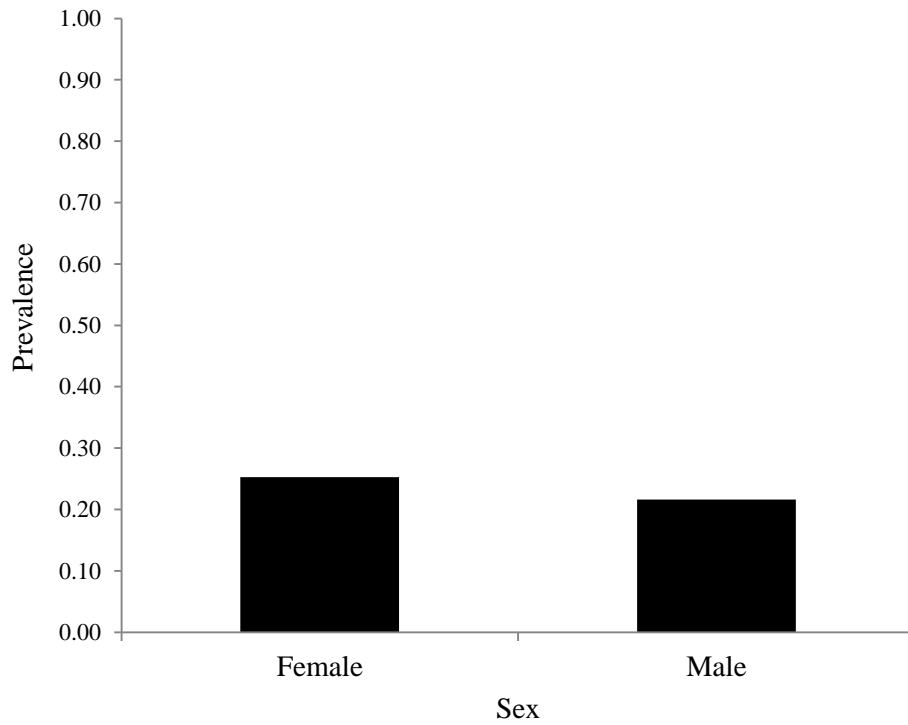
Figure 1.1 Combined *Ichthyophonus* prevalence by sex for 2012 and 2013; there was no difference in overall prevalence between females and males.
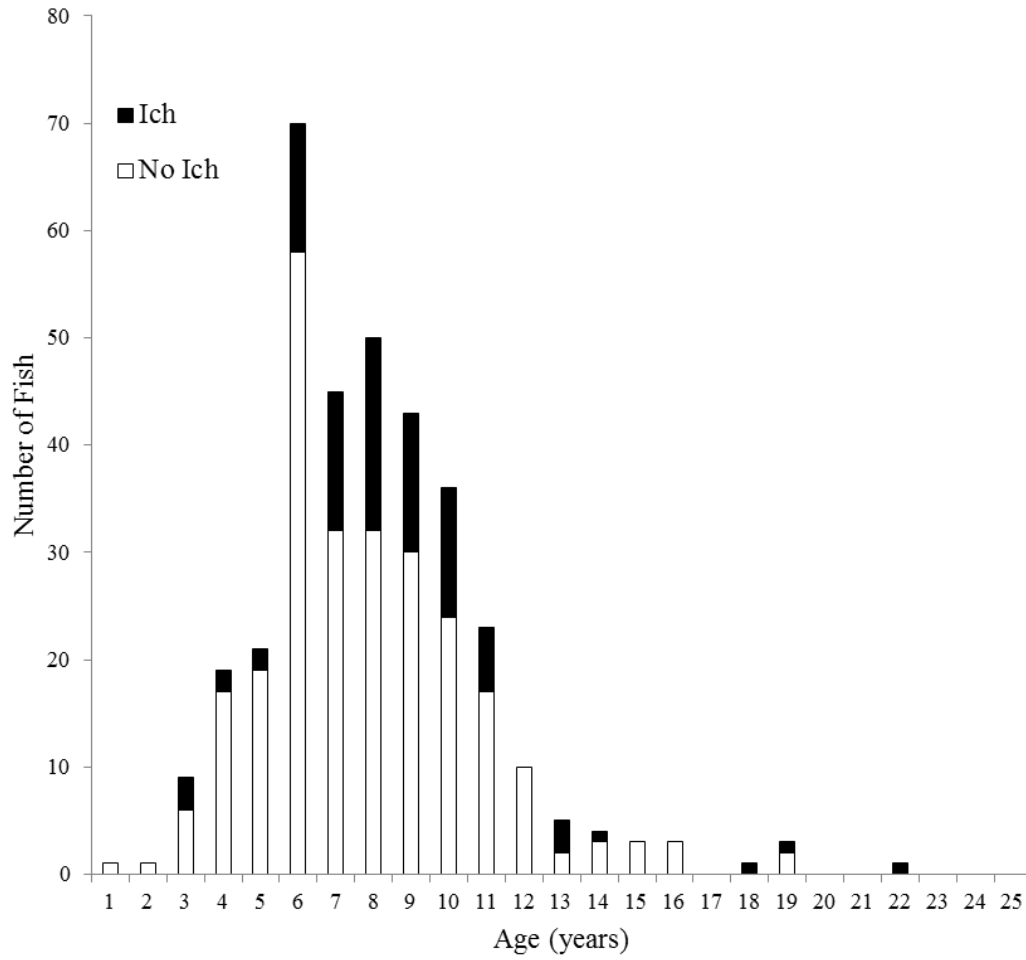
Figure 1.2 *Ichthyophonus* distribution in females by age. The youngest sampled positive was 3 years, while the oldest was 22 years.
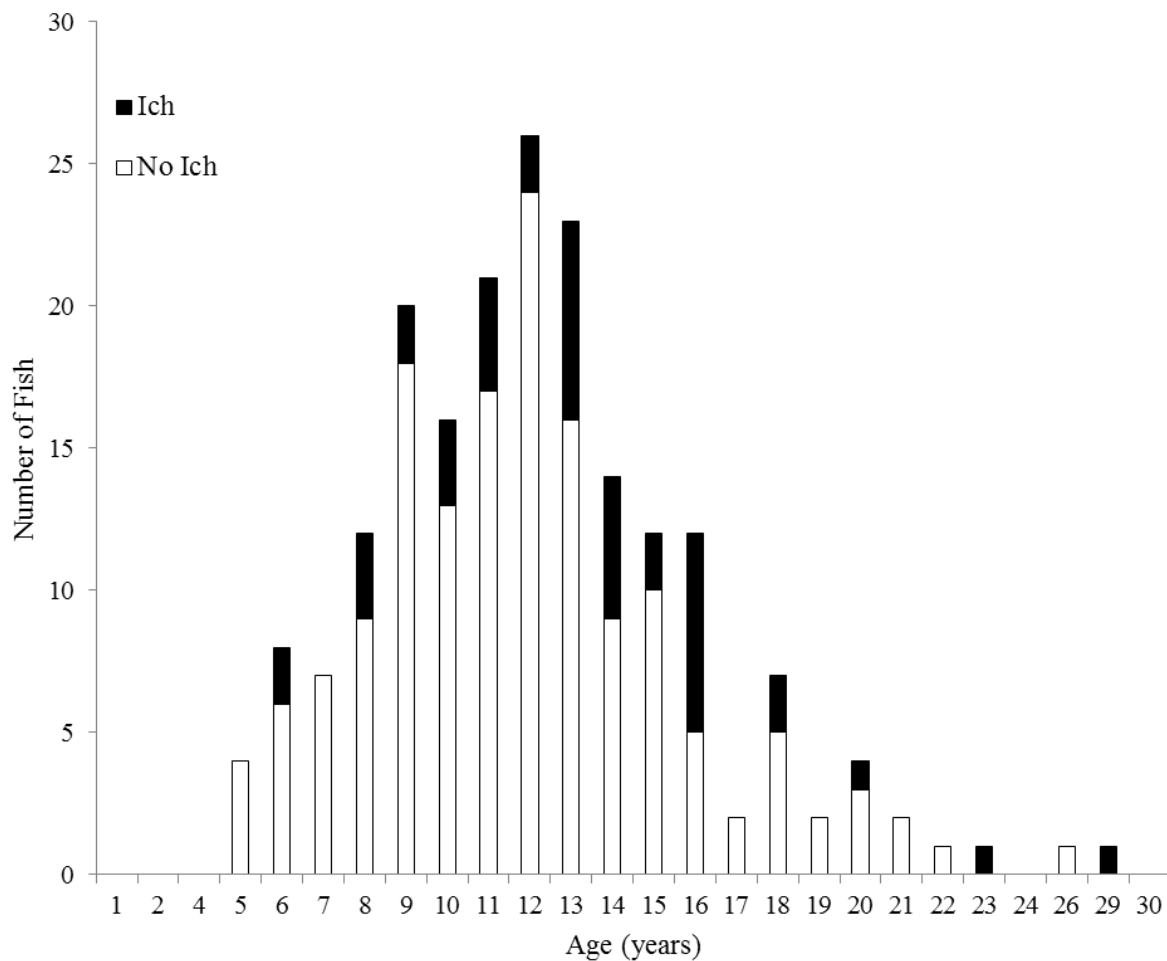
Figure 1.3 *Ichthyophonus* distribution in males by age. The youngest sampled positive was 6 years old, while the oldest was 9 years.

Age

Ichthyophonus was present in all age classes of halibut tested, and was positively correlated with age (y= 0.02(Age), $R^2 = 0.31$, p < 0.005, Figure 1.4). This correlation strengthened when data were truncated to exclude only age classes containing n $\geq$ 10 (y= 0.021(Age), $R^2 = 0.88$, p < 0.001, Figure 1.5).
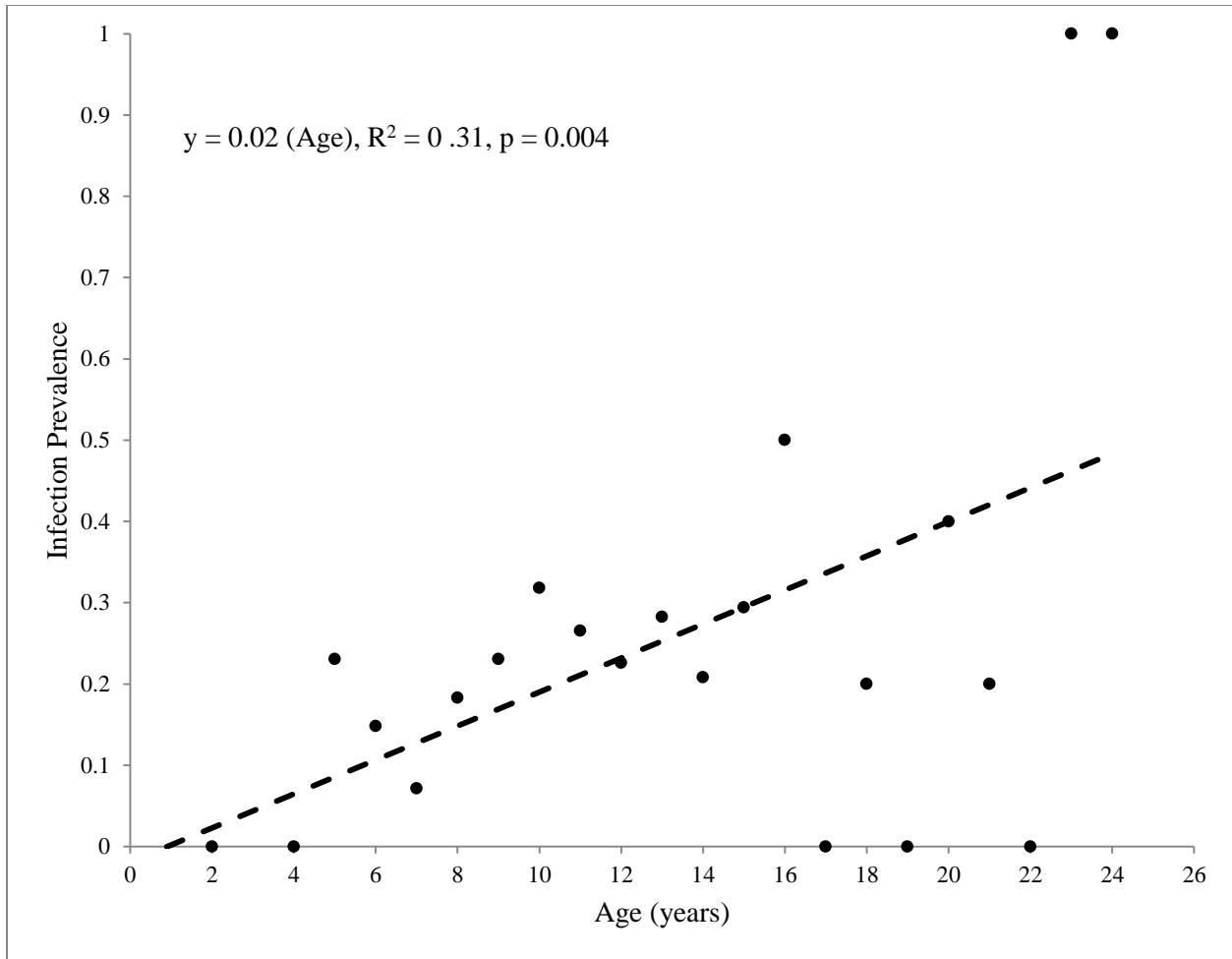
Figure 1.4 *Ichthyophonus* prevalence by age with linear regression. Age accounts for 31% of the variability in *Ichthyophonus* prevalence.
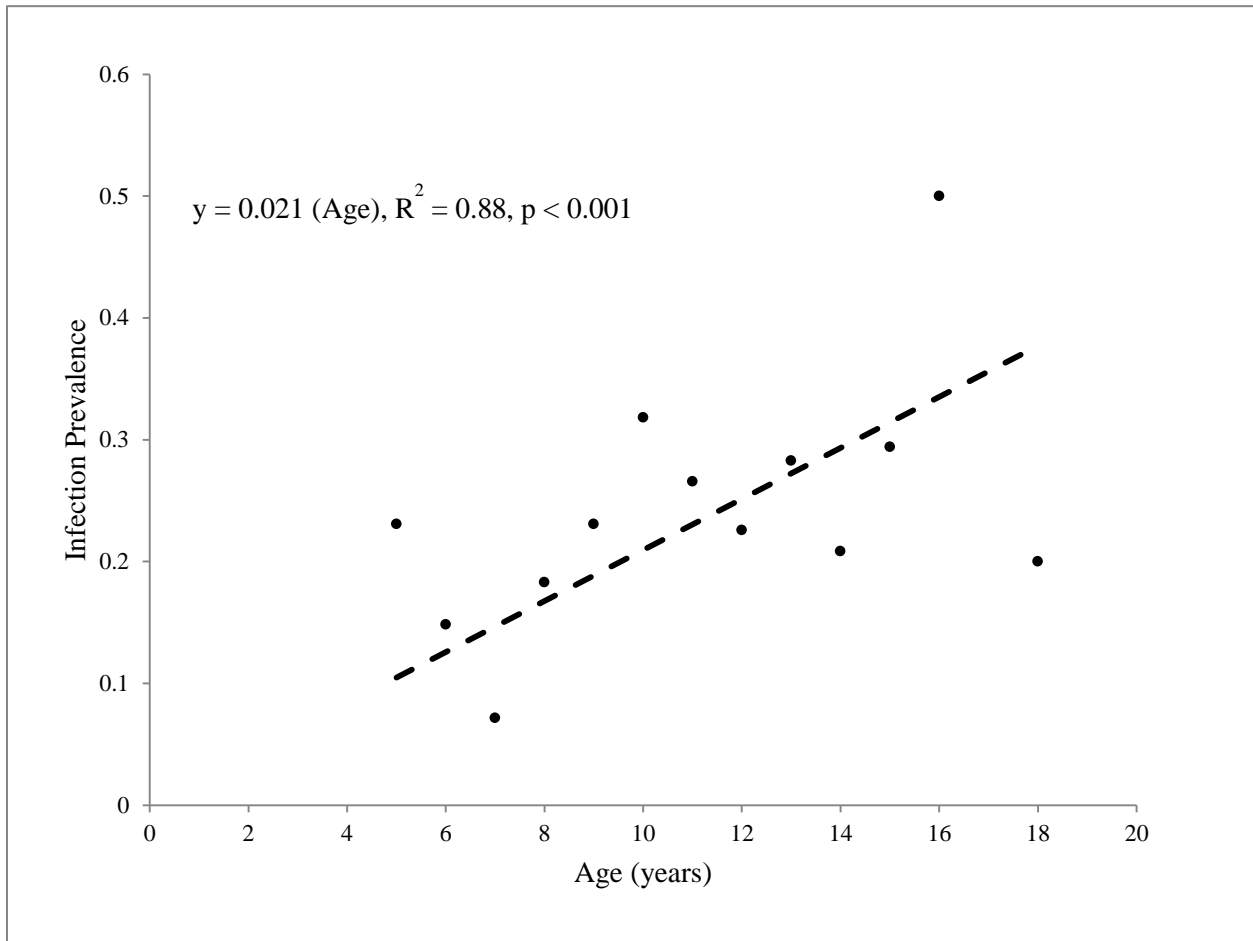
Figure 1.5 *Ichthyophonus* prevalence by age, with linear regression; data were truncated to exclude samples with n<10. When data were truncated age accounted for 88% of the variability in *Ichthyophonus* prevalence.

Size at Age

Contrary to previous studies on experimentally infected herring, Pacific halibut size at age was
not affected by *Ichthyophonus* infection status. Among males, mean size at age was similar
between infected and uninfected individuals (Table 1.2, 1.3). Additionally, mean size at age was
also similar between infected and uninfected females except those in the 7-year age class (N= 2,
N= 19, P = 0.016, χ^2, Figures 1.6 and 1.7). However, we note that there were only 2 infected 7-
year old fish. Within the 10 cm fork length size bins there was a wide range of age classes
(Figures 1.8 – 1.11); we found no significant difference in mean age at size between infected and
uninfected males or females.

Table 1.2 Comparison of mean size-at-age between *Ichthyophonus* positive and negative females. The only significant difference in mean size at age was among 7 year olds.

	No Ich			Ich					
Age	N	Mean L	SD L	N	Mean L	SD L	P value	df	t-test
5	6	58.83	5.31	3	56.33	6.11	0.58	3.6	0.60
6	17	73.76	12.30	2	66.50	3.54	0.12	5.2	1.87
7	19	77.79	7.71	2	72.00	1.41	0.02*	11.1	2.85
8	58	80.78	10.11	12	85.92	19.92	0.40	12.2	-0.87
9	32	85.91	13.35	13	82.54	5.14	0.23	43.0	1.22
10	32	90.22	16.05	18	87.06	16.89	0.52	33.9	0.65
11	30	93.07	15.96	13	94.54	17.17	0.80	21.4	-0.26
12	24	104.33	27.56	12	112.25	28.22	0.43	21.6	-0.80
13	17	109.35	30.86	6	102.83	18.78	0.55	14.9	0.61
15	2	125.50	27.58	3	163.33	51.07	0.36	3.0	-1.07

Table 1.3 Comparison of mean size-at-age between *Ichthyophonus* positive and negative males. There was no significant difference in mean size at age among any age class.

	No Ich			Ich					
Age	N	Mean L	SD L	N	Mean L	SD L	P value	df	t-test
6	6	64.00	7.92	2	63.00	4.24	0.83	3.7	0.23
8	9	71.56	9.03	3	75.67	6.81	0.45	4.6	-0.83
9	18	73.78	5.61	2	80.00	4.24	0.25	1.4	-1.90
10	13	76.31	4.64	3	68.33	5.03	0.09	2.8	2.51
11	17	77.18	6.07	4	74.25	3.20	0.21	9	1.35
12	24	77.04	6.00	2	74.50	3.54	0.48	1.5	0.91
13	16	76.38	3.96	7	76.29	2.87	0.95	15.8	0.06
14	9	76.67	4.42	5	73.00	4.30	0.17	8.6	1.51
15	10	81.30	4.90	2	82.00	1.41	0.72	7.1	-0.38
16	5	80.60	3.36	7	79.00	4.32	0.49	9.9	0.72
18	5	82.80	4.92	2	83.00	1.41	0.94	5	-0.08
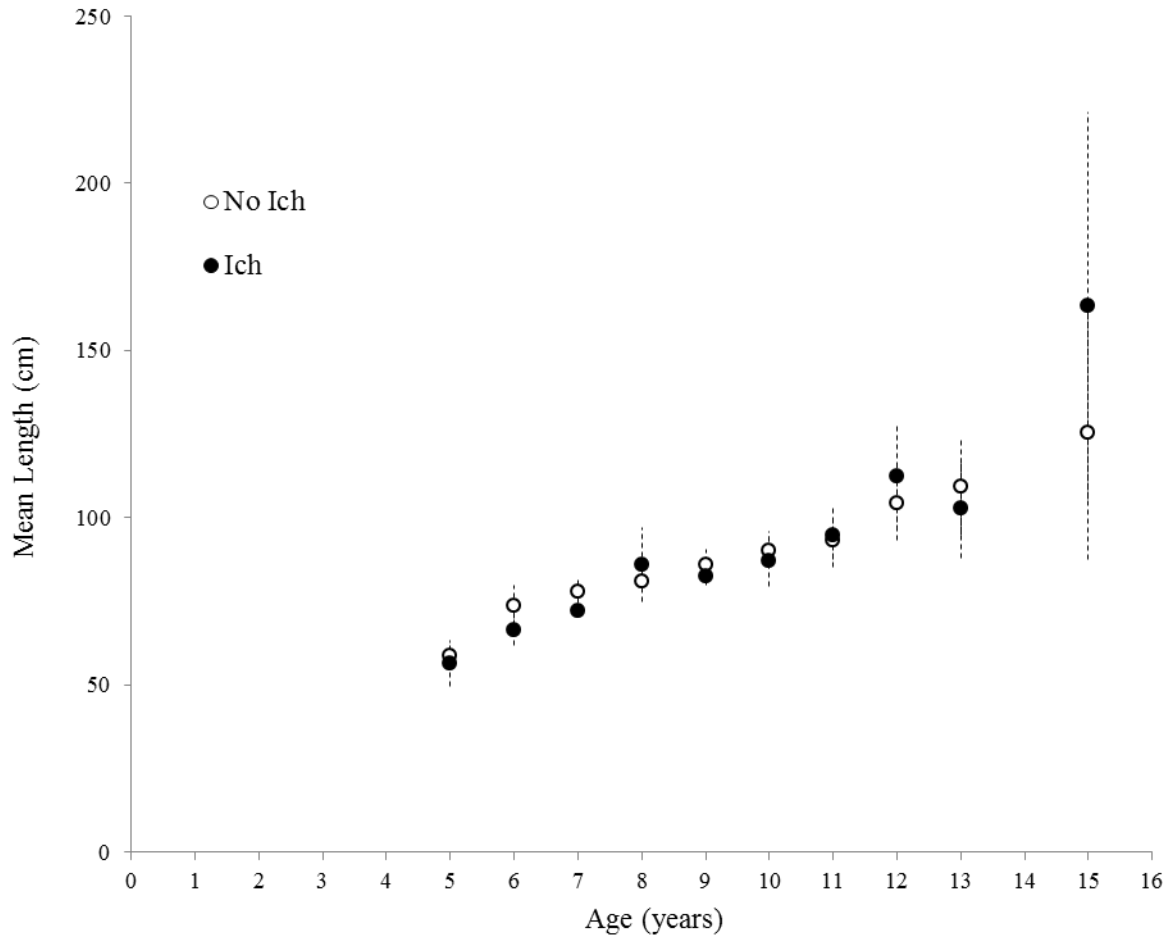
**Figure 1.6 Comparison of mean size-at-age between *Ichthyophonus* positive and negative females. There is no significant difference between mean size and age among infected and uninfected females except for 7-year old fish (N (infected) = 2, N (uninfected) = 19, df= 11.1, p = 0.016).**
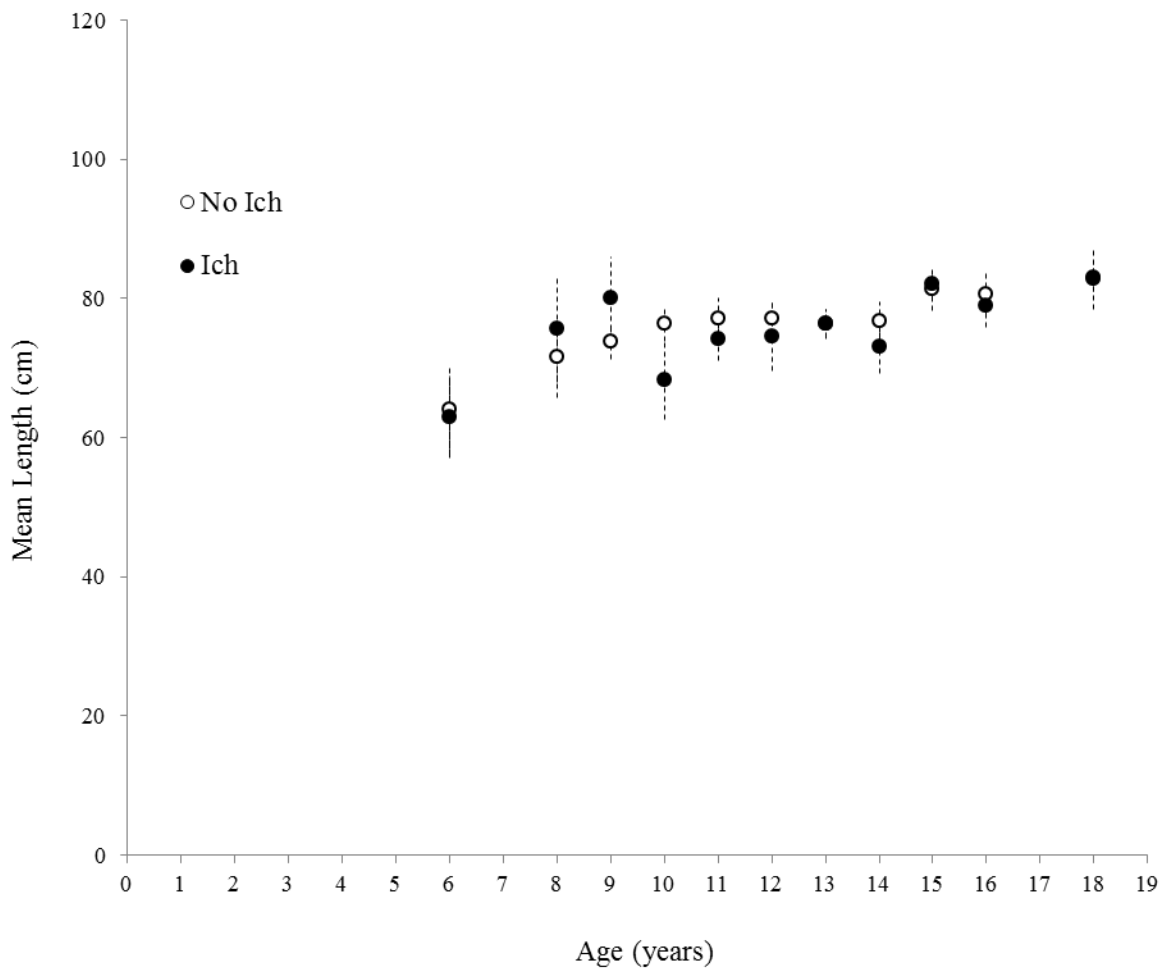
Figure 1.7 Comparison of mean size-at-age between *Ichthyophonus* positive and negative males with 95% confidence intervals. There is no significant difference between mean size at age among infected and uninfected males (see table 1.3).
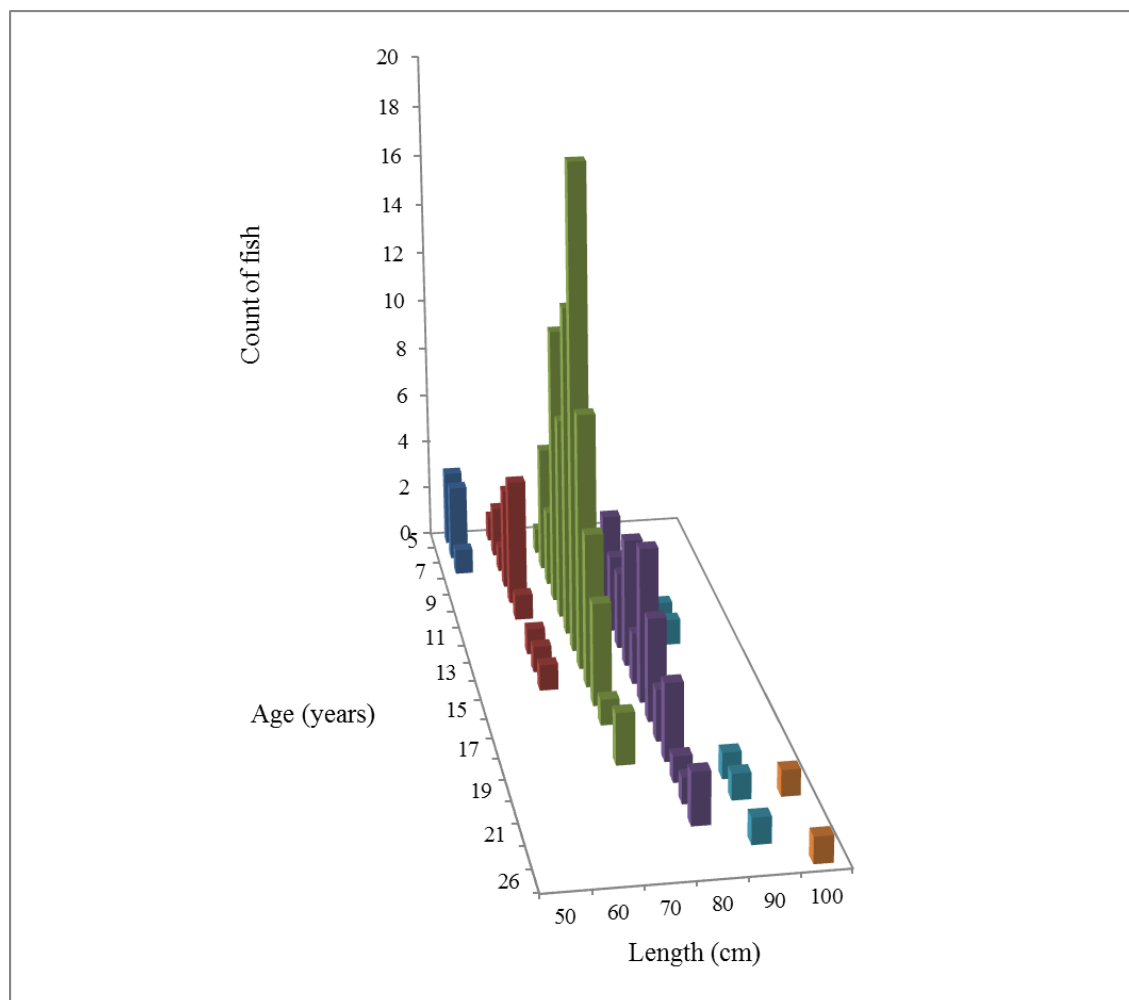
Figure 1.8 *Ichthyophonus* negative male age class distribution among 10 cm fork length bins. There is a wide array of ages among 10 cm fork length bins, with the widest range among 80 – 90 cm sampled fish.
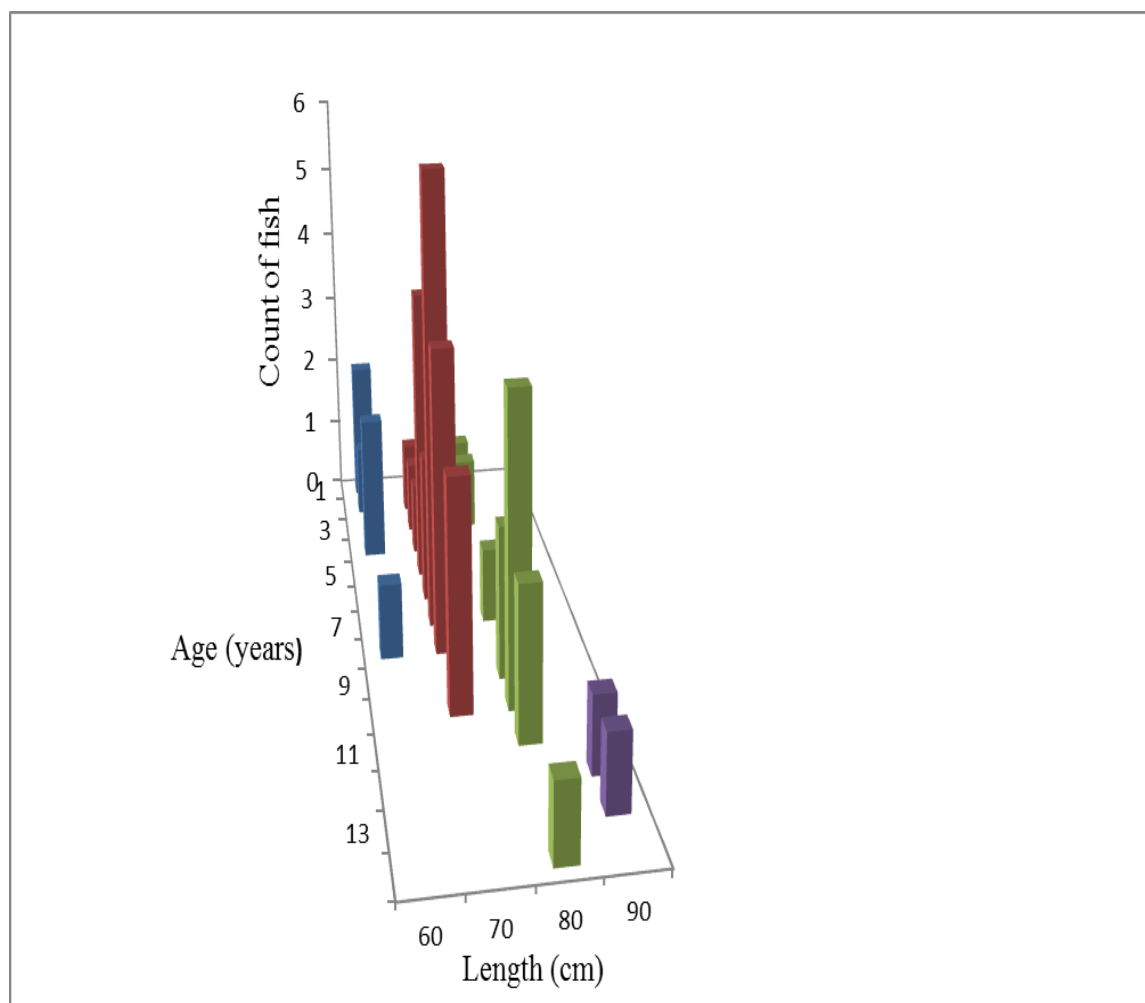
Figure 1.9 *Ichthyophonus* positive male age class distribution among 10 cm fork length bins. There is a wide array of ages among 10 cm fork length bins, with the widest range among 70 – 80 cm sampled fish.

Figure 1.10 *Ichthyophonus* **positive female age class distribution among 10 cm fork length bins. There is a wide array of ages among 10 cm fork length bins, with the widest range among 80 – 90 cm sampled fish.**

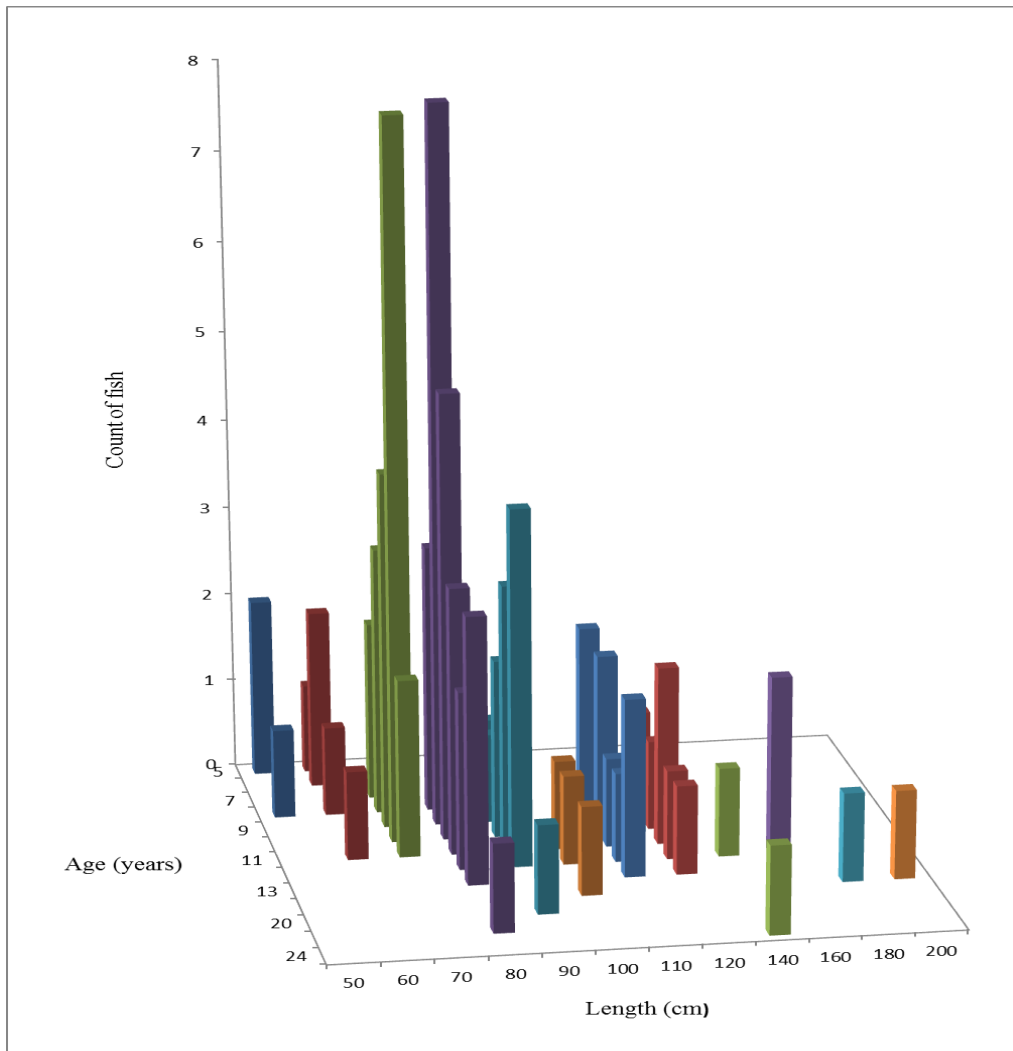**Figure 1.11 *Ichthyophonus* negative female age class distribution among 10 cm fork length bins. There is a wide array of ages among 10 cm fork length bins, with the widest range among 80 – 90 cm sampled fish.**
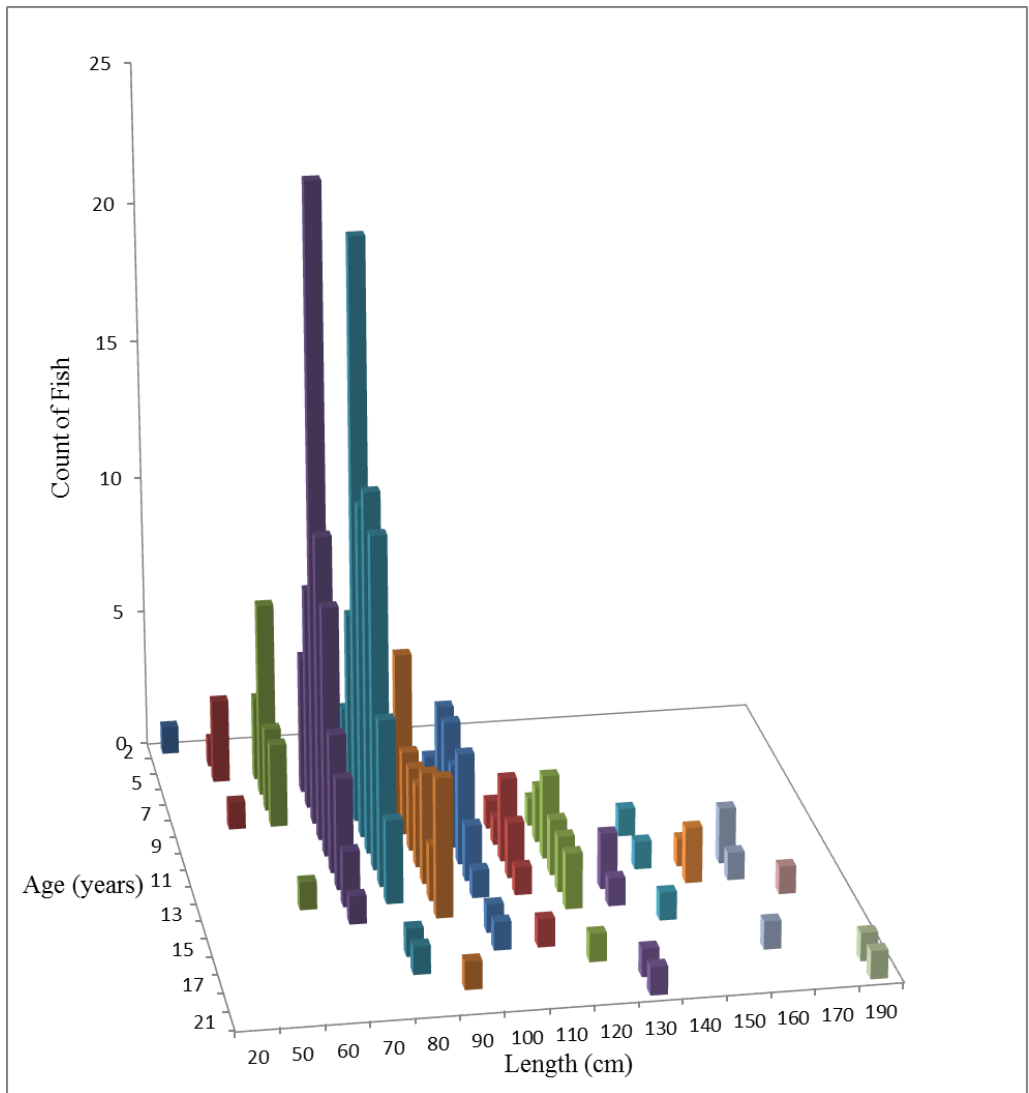
1.4 DISCUSSION

Over two years we found *Ichthyophonus* in all sizes, ages, and in both sexes in 26% of the 563 Pacific halibut (*Hippoglossus stenolepis*) we tested. There was a 7% increase in prevalence from 2012 to 2013, suggesting that annual infection prevalence in Pacific halibut is variable. Interannual variability in *Ichthyophonus* prevalence likely reflects interannual variability in other ecological variables. Therefore, ongoing monitoring and determination of ecological drivers is warranted.

We only found *Ichthyophonus* within the heart, with no evidence of infection in the liver, kidney, or spleen tissues and none of the fish we examined displayed overt clinical signs of infection (e.g., white nodules in soft tissues). *Ichthyophonus* affects different host species, and even individuals of the same species, in different ways. Rand (1994) found creamy-white lesions on the liver and kidney tissues of yellowtail flounder (*Limanda ferruginea*), yet none on the heart. They suggest this may be due to varying strains of *Ichthyophonus*, host immune responses, and cryptic species – a genetically distinct species that looks and manifests in a similar manner. Halos *et al*. (2005) examined the variation in infection among Puget Sound rockfish (*Sebastes emphaeus)*, Yukon River Chinook salmon (*Oncorhynchus tshawytscha)*, and Pacific herring (*Clupea pallasii*,). In rockfish *Ichthyophonus* cultured positive in the heart and liver tissues, yet no tissue samples displayed overt clinical signs of infection. In Chinook salmon and herring, however, gross clinical signs (e.g. internal lesions) were visible, primarily in the heart and kidney. Genetic comparisons of the highly conserved 18S ribosomal DNA region indicated that *Ichthyophonus* isolated from all three hosts subscribed to the same genetic type (Halos *et al*. 2005). However, this genetic type was different than that previously reported in other rockfishes throughout the NE Pacific. These apparent differences in *Ichthyophonus* types between host

species likely involve some combination of parasite life cycle differences between the genetic types that are realized through differences in host diets. It is unclear why only halibut hearts are affected but on-going genetic research by USGS (Gregg and Hershberger Pers Comm) and diet studies (Webster M.Sc. Thesis) may provide insight.

Similar to other species, such as Yukon River Chinook salmon and rainbow trout (Kocan *et al.* 2004), we found that *Ichthyophonus* was prevalent in male and female Pacific halibut at similar levels (22-25%; Figure 1.1). This aligns with observations on Pacific halibut from the Bering Sea, Prince William Sound, and Oregon coast (NPRB RARA, project #1015, 2013).

The prevalence of *Ichthyophonus* increased directly with halibut age, approximately 2% per year between the ages of 2 and 16, with infection prevalence reaching 50% in age 16 yr. cohorts (Figure 1.3). Hershberger *et al.* (2002) hypothesized that an analogous demographic pattern in Pacific herring resulted from an increased probability of single or multiple exposures as herring age. In their study of wild Pacific herring, prevalence increased from 12% among juveniles to 58% in individuals older than 6 years. The current paradigm is that once fish contract *Ichthyophonus* there is no recovery (McVicar 2011). If the disease does not cause mortality, the host continues to carry the parasite throughout its life and is a potential horizontal vector. It is not established why some fish die soon after contracting the disease and why others carry the parasite apparently unaffected. Currently, there is no evidence that *Ichthyophonus* causes death in Pacific halibut.

Unlike studies on rainbow trout and herring (Kramer-Schadt, 2010, Kocan *et al.*, 2010) we found no evidence that *Ichthyophonus* infection influenced growth rates in Pacific halibut (Figures 1.4 and 1.5). Conversely, concurrent research using the fish analyzed in this study indicates that they are larger and faster growing than halibut caught in other fishing areas within Alaska (Webster

M.Sc. thesis). Within the 10 centimeter size bins, there was a wide range of ages for both *Ichthyophonus* positive and negative fish. The largest age difference in *Ichthyophonus* positive fish was in the 80-89 cm group, from 8 to 29 years, and for *Ichthyophonus* negative fish was in the 100-109 cm group, from 6 to 26 years. This wide variation in size at age may also be masking any *Ichthyophonus*-related growth impacts due to low sample size within any size-age group.

Ichthyophonus research in the Cook Inlet Pacific halibut population – as well as preliminary infection data from the northern Bering Sea, Prince William Sound, and Oregon Coast – has not shown adverse effects on mean size at age, and indicates equal distribution between sexes. Currently, our understanding of how *Ichthyophonus* impacts halibut is nascent. This is due, in part, to the use of binary culture-based methods which indicate whether or not a fish is infected. This approach is useful for determining initial infection rates but cannot account for individual fish effects. In the next chapter of this thesis I develop and test a method for isolating *Ichthyophonus* schizonts to assess the parasite load in individual fish.

CHAPTER 2

ISOLATING AND QUANITFYING *ICHTHYOPHONUS* SCHIZONTS IN PACIFIC

HALIBUT (*HIPPOGLOSSUS STENOLEPIS*) HEARTS BY PEPSIN DIGESTION

2.1 INTRODUCTION

Marine epidemics are increasing in both in number and frequency (Harvell et al. 1999; Sherman 2000). This increase, particularly within the past 30 years, has presented scientists and policy makers with opportunities to explore potential adverse impacts on ecological and economic systems. This work revealed that one low host specific cosmopolitan parasite, *Ichthyophonus hoferi,* has caused multiple epizootic outbreaks among many commercially important fishes, both marine and freshwater, resulting in negative economic impacts (Kramer-Schadt *et al*. 2010; Kocan et al 2010; McVicar 2011). *Ichthyophonus hoferi* is known to infect more than 100 fish species worldwide including Atlantic and Pacific herring (*Clupea pallasii*), mackerel (*S. scombrus*), yellowtail flounder (*Pleuronectes ferruginea*), Chinook salmon (*Oncorhynchus tshawytscha*), and American shad (*Alosa sapidissima*) (Burge *et al.* 2014; McVicar, 2011; Kocan *et* al., 2004; Kramer-Schadt *et al*., 2010; Kocan et al., 2010).

Ichthyophonus causes the disease *Ichthyophoniasis* in both cultivated and wild fish. Most *Ichthyophonus* reports are from pelagic waters and estuaries but some freshwater outbreaks, particularly in aquaculture, have been reported (McVicar 2011). Freshwater infections have been linked to the practice of feeding raw infected feed obtained from marine fish (McVicar, 2011, Sindermann and Chenoweth, 1993). Anadromous fish, such as Atlantic salmon (*Salmo salar*) and most recently Chinook salmon are also known to harbor the parasite (Kocan *et al*., 2004). External signs include skin roughening (known as the "sand paper" effect), black papules, ulceration (particularly in the lateral muscle), and wasting of musculature (Kocan *et al*., 2010). Highly vascularized organs are primarily infected and can display white nodules, consisting largely of the multinucleated schizonts and surrounding granulomatous (Kocan *et al*., 2006).

There are multiple methods for detecting *Ichthyophonus* infections. However, prevalence rates

can vary greatly among fish populations and individuals depending on which method is used

(McVicar, 2011). Macroscopic, or visual detection of *Ichthyophonus* infection by clinical signs

such as ulcers and "sandpaper skin," is useful during field experiments to establish

Ichthyophonus epizootics. This technique, however, can grossly underestimate infection

prevalence, as overt signs typically only present during extended and high levels of infection. It

is also probable that the specific host does not demonstrate any overt physical signs, therefore

masking infection (McVicar 2011). Microscopic evaluations of tissue squashes are also

employed during field studies on *Ichthyophonus*, though accurate prevalence rates are often

difficult to obtain due to the uneven distribution throughout host organs and tissues (Kocan *et al*.

2011, McVicar 2011).

Histological evaluation is a commonly used technique to confirm the identity of *Ichthyophonus*

in host tissues and to assess cellular damage, but it is not always a suitable technique to

accurately screen populations for infection due to the uneven distribution of the parasite within

the host (Kocan *et al*. 2011, McVicar 2011). Commonly used histological stains include

haematoxylin and eosin (H & E) and periodic acid-Schiff (PAS). H & E lightly stains the

cytoplasm of the cell outlining internal organelles and nuclei and is particularly useful for

assessing host cellular immune response to infection, and PAS reacts with the polysaccharides

within the outer walls of the *Ichthyophonus* cell. Neither stain is specific to *Ichthyophonus*; as a

result morphologically similar parasites can easily be mis-identified as Ichthyophonus. Unlike

the non-specific stains, Chromogenic *in situ* hybridization binds specifically to unique regions of

the *Ichthyophonus* genome; successful binding is accompanied by a color reaction, thereby

providing confirmatory diagnosis from histological sections. However, the CISH is intended as

a confirmatory laboratory diagnostic tool rather than a viable field surveillance tool.

Polymerase chain reaction (PCR) techniques using *Ichthyophonus*-specific primers are useful in

revealing the presence of parasite nucleic acid and confirming presumptive diagnoses, but cannot

determine whether live parasites are present. Currently, the most accurate technique for

detecting *Ichthyophonus*, in living and recently deceased hosts, is the *in vitro* culture of tissue

explants in Minimum Eagle's Medium (MEM) (Hershberger 2012, McVicar 2011). This has

proven the most sensitive method, and thus, is most frequently used to determine *Ichthyophonus*

infections within populations (Hershberger 2012).

Although the culture technique is currently the most accurate at detecting infection, it does not

provide any information regarding parasite load or infection intensity. Hence, while this is

helpful to determine infection prevalence, it does not account for or measure individual

differences in parasite load. Kocan *et al.* (2011) explored this difference among *Ichthyophonus*

density by isolating and quantifying schizonts within external ulcers and the underlying muscle

of experimentally infected Pacific herring and determined a wide range and uneven distribution

of schizonts. While Kocan *et al.* (2011) ultimately used glycerine for the tissue clearing, another

method used for parasite detection in other fish, (e.g., whirling disease in salmonids) involves

pepsin digestion (USFWS, 2014). Pepsin digestion is used to isolate parasites that are found

within organs, muscle tissue, and cartilage (USFWS, 2014). This method is useful for detecting

certain parasites at low level infections and uneven distribution among organs, muscle, and

cartilage. By using pepsin digestion, infection prevalence can be assessed by observing parasites

that are liberated from the tissues; additionally, the total number of parasites scattered throughout

the tissues can be quantified, therefore allowing for the determination of infection intensity and load.

The objective of this study was to develop and test a pepsin digestion technique capable of isolating and enumerating *Ichthyophonus* stages from the tissues of Pacific halibut *(Hippoglossus stenolepis).*

2.2 METHODS AND MATERIALS

Sampling

Halibut were sampled in the port of Homer in collaboration with the Alaska Department of Fish and Game port-sampling program. Located in lower Cook Inlet, Homer supports the largest Pacific halibut sport fishery in the United States (Meyer and Powers 2013). Most of the local fishers and charter fishing operations fillet their catch at fish processing stations located at the port. At these stations ADFG staff interview fishers and sample the catch. Once filleted, the carcasses are discarded in dumpsters, ultimately ground and dumped at sea. Samples were collected from carcasses, prior to their disposal in the dumpsters; biological data from each fish included fork length (cm), sex (2012 Field Procedure Manual for the Southcentral Alaska Halibut and Groundfish Harvest Assessment Program of ADF&G), and age (determined from sagittal otoliths). Our aim was to sample at least 30 male and 30 female halibut in as many 10 cm fork length size bins as possible (< 39 cm, $40 - 49$ cm, $50 - 59$ cm, etc.)

Infection status was confirmed by explant culture of heart samples (approximately 0.5 g each) in MEM. The remaining heart tissues were frozen at -20°C for future pepsin digestion. If a fish

cultured positive for *Ichthyophonus*, the remainder of the previously-frozen heart was digested in pepsin to enumerate parasite load, described as the total number of schizonts per gram of heart tissue (n_s/tissue weight (g)). Pepsin digestion was chosen because it mimics stomach conditions, where consumed tissue are digested, but *Ichthyophonus* life stages are able to survive due to a surrounding non-cellular multilaminate membrane (Kocan *et al*. 2013, McVicar 2011). Pepsin solution was made by mixing 5 mg of pepsin and 5 mL of hydrochloric acid (12 M) in 1 L of distilled water until all solids were dissolved following USFWS standard procedures (USFWS 2014).

The pepsin digestion protocol was initiated by thawing the frozen hearts in a room temperature water bath. Hearts were then weighed on an Ohaus balance to the nearest 0.1g. The digestion process was facilitated by cutting each heart into smaller sections and transferring the tissues to a beaker containing pepsin solution (1:20 w/v), that was heated to 55 C^o, and mixed. Previous digestive studies on whirling disease suggest 37° C but the stir plate manufacturers stated that the surface of the plate is approximately 15 C^o warmer than the sample being processed so the temperature was increased. Heat was discontinued when all heart tissue was digested. The harvested Ichthyophonus stages in the pepsin solution were then stained with safranin dye solution (approximately 0.5 mL to every 100 mL solution for 30 seconds.   The stained *Ichthyophonus* life stages were then harvested by vacuum filtration onto a 1 μm glass fiber filter paper. The dyed filters were then placed on a holding tray labeled with the sample number. After the filter paper dried (approximately one day) it was examined under 40x magnification on a Leica M60 stereomicroscope and all visible schizonts were counted to enumerate the number of parasite schizonts.

To have a standardized method for quantifying *Ichthyophonus*, we followed the same criteria

outlined in histological evaluations as described by McVicar (2011). Schizonts were defined as

spherical bodies ranging in size from 10 – 250 μm. There were no germinating bodies and or

hyphae observed or counted. For comparison, schizonts were taken directly from the media

culture and dyed with 1% safranin.

Analysis

Parasite load was calculated by counting the number of visible *Ichthyophonus* schizonts and

dividing that by the total heart weight. A t-test was used to compare loads between males and

females. Linear regression was used to examine the relationship between *Ichthyophonus*

infection and age. It was determined in chapter 1 that infected and non-infected fish are not

different in size-at-age so the relationship between size-at-age and intensity was not evaluated.

Total schizonts were calculated for the entire heart by multiplying 0.5 (amount of heart taken for

culture) by the specific intensity of that heart, then adding the number of schizonts observed.

Thirty hearts that did not culture positive were also digested to test the effectiveness of both

pepsin digestion and the culture method.

2.3 RESULTS

Sampling

A total of 563 Pacific halibut (364 females, 199 males) were sampled in 2012 and 2013 with 134

culturing positive for *Ichthyophonus*. Of these, 96 hearts were digested for intensity. Schizonts

were not detected in any of the randomly selected culture-negative hearts (n=30) after pepsin

digestion.

Sex

Of the 96 digested hearts there was no significant difference between average intensity in

females and males (Table 2.1, Figure 2.1, N = 69, N = 27, P = 0.088, χ^2)

Table 2.1 Average *Ichthyophonus* intensity by sex for 2012 and 2013 combined

| Sex	N	Mean I	95% CI ($\pm$)	Sd
Female	69	32.6	5.84	22.96
Male	27	23.9	6.24	15.78
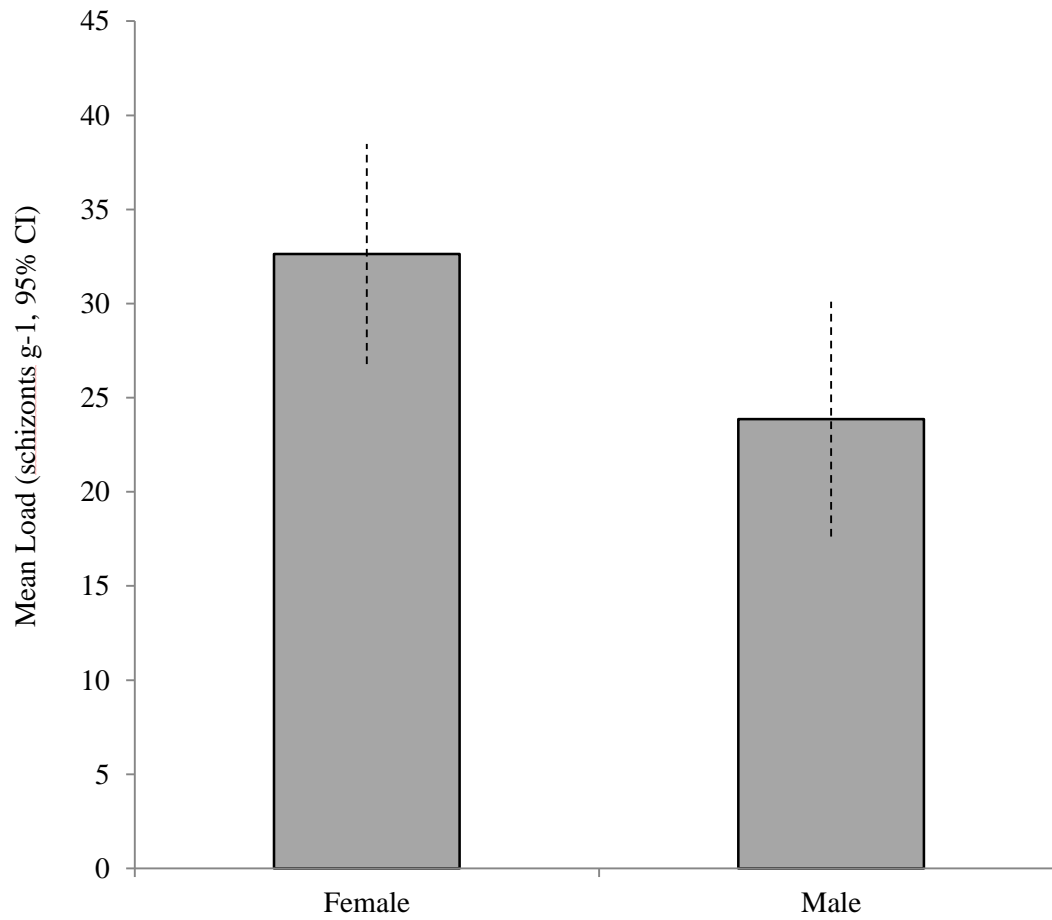
Figure 2.1 Average *Ichthyophonus* load by sex for 2012 and 2013 combined, with 95% confidence interval. There is no difference between mean *Ichthyophonus* load between males and females.

Age

Ichthyophonus intensity varied among all age classes with more than one sample and had no correlation with age (Figure 2.2, y = -0.95(Age) +40, R^2 = 0.03, P = 0.113). When the average intensity was taken per age class there was a slight negative correlation (Figure 2.3, y = -1.21(Age) + 47.8, R^2 = 0.25, P = 0.04). When data were truncated to exclude age classes with less than 7 samples the correlation strengthened slightly (Figure 2.4, y = -3.78(Age) + 43, R^2 = 0.31, P = 0.08).
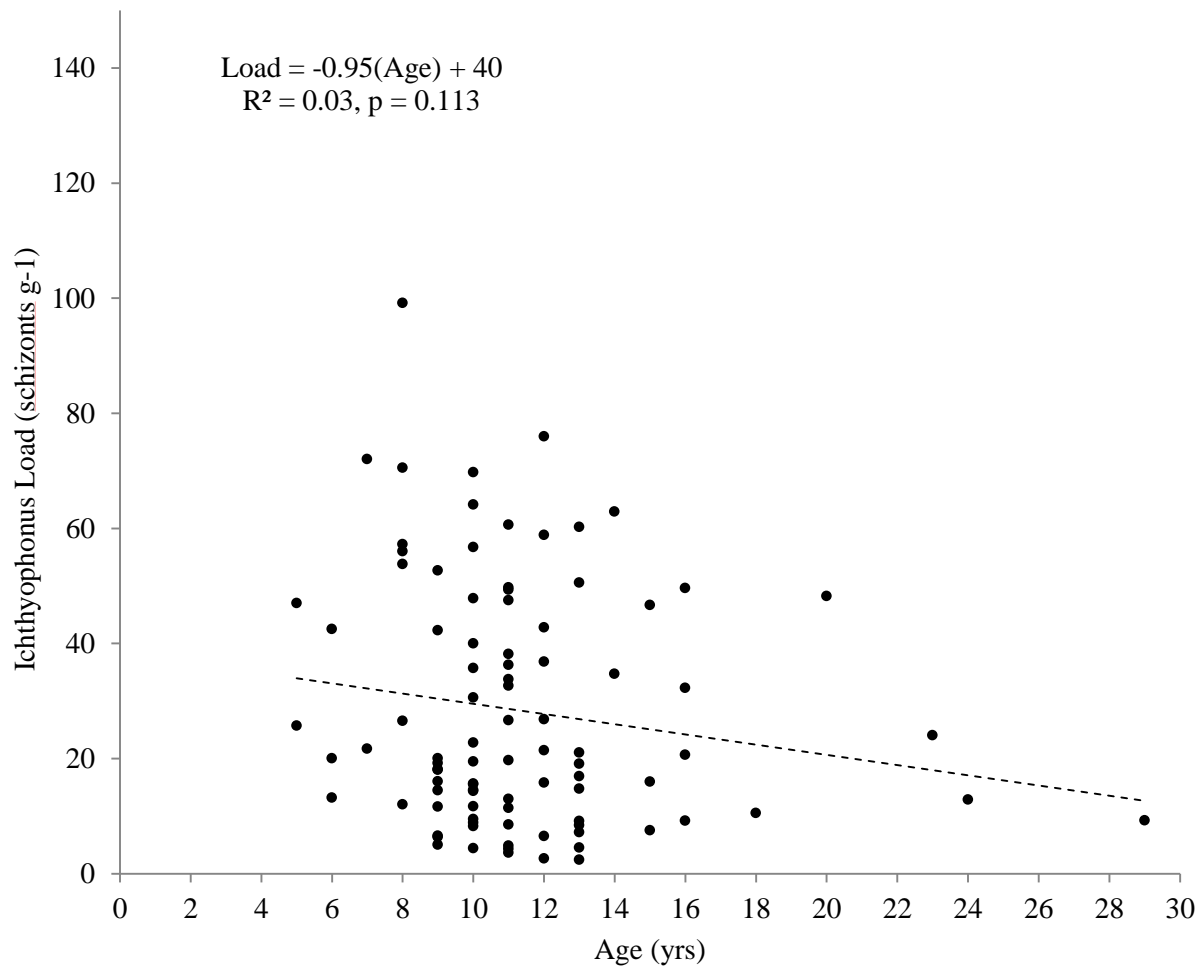
Figure 2.2 *Ichthyophonus* **tissue burdens for all age classes and males and females. Age does not account for variability of *Ichthyophonus* load.**
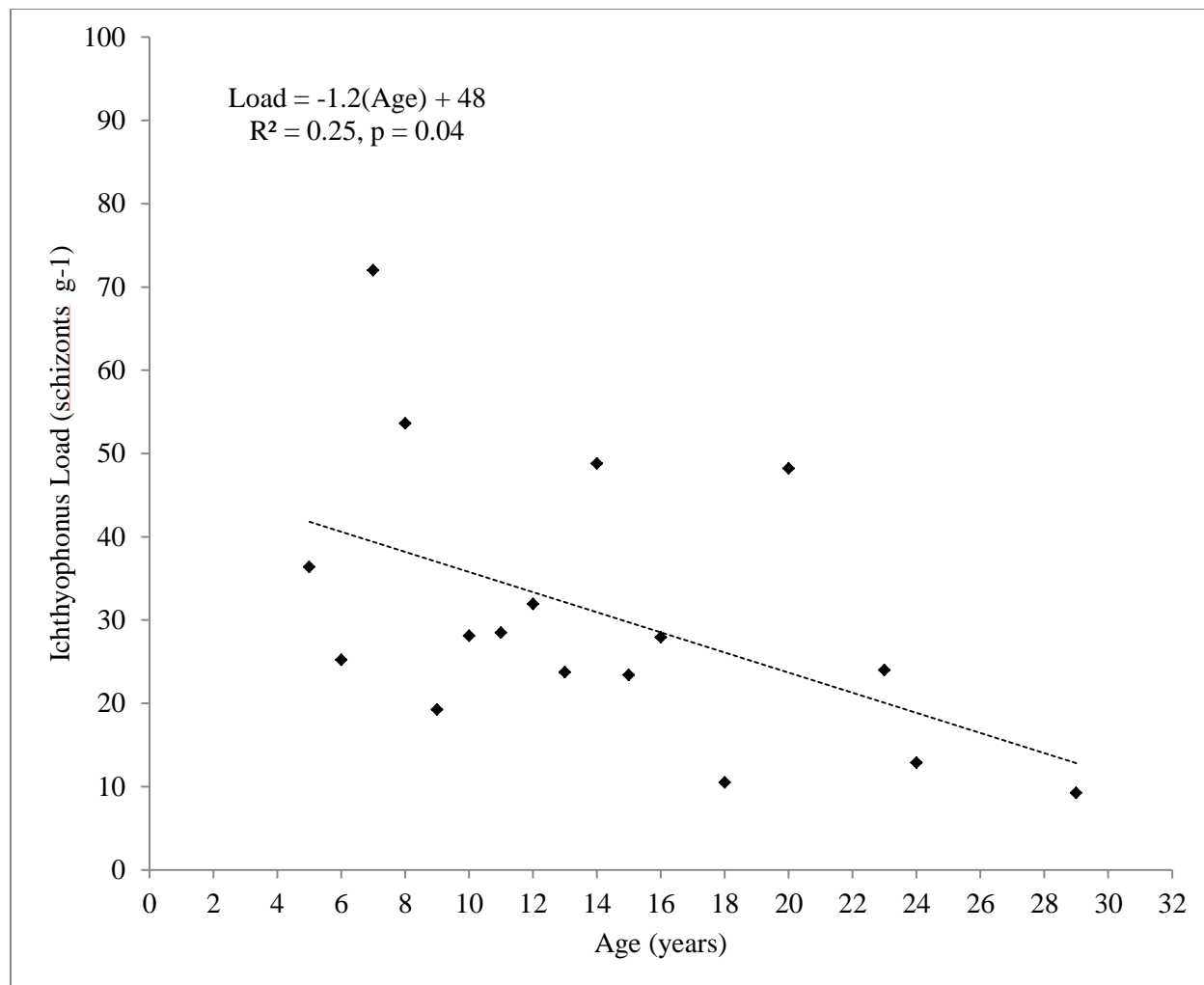
Figure 2.3 Average *Ichthyophonus* tissue burdens for all age classes and males and females.

Age accounts for 25% of the variability in *Ichthyophonus* load.
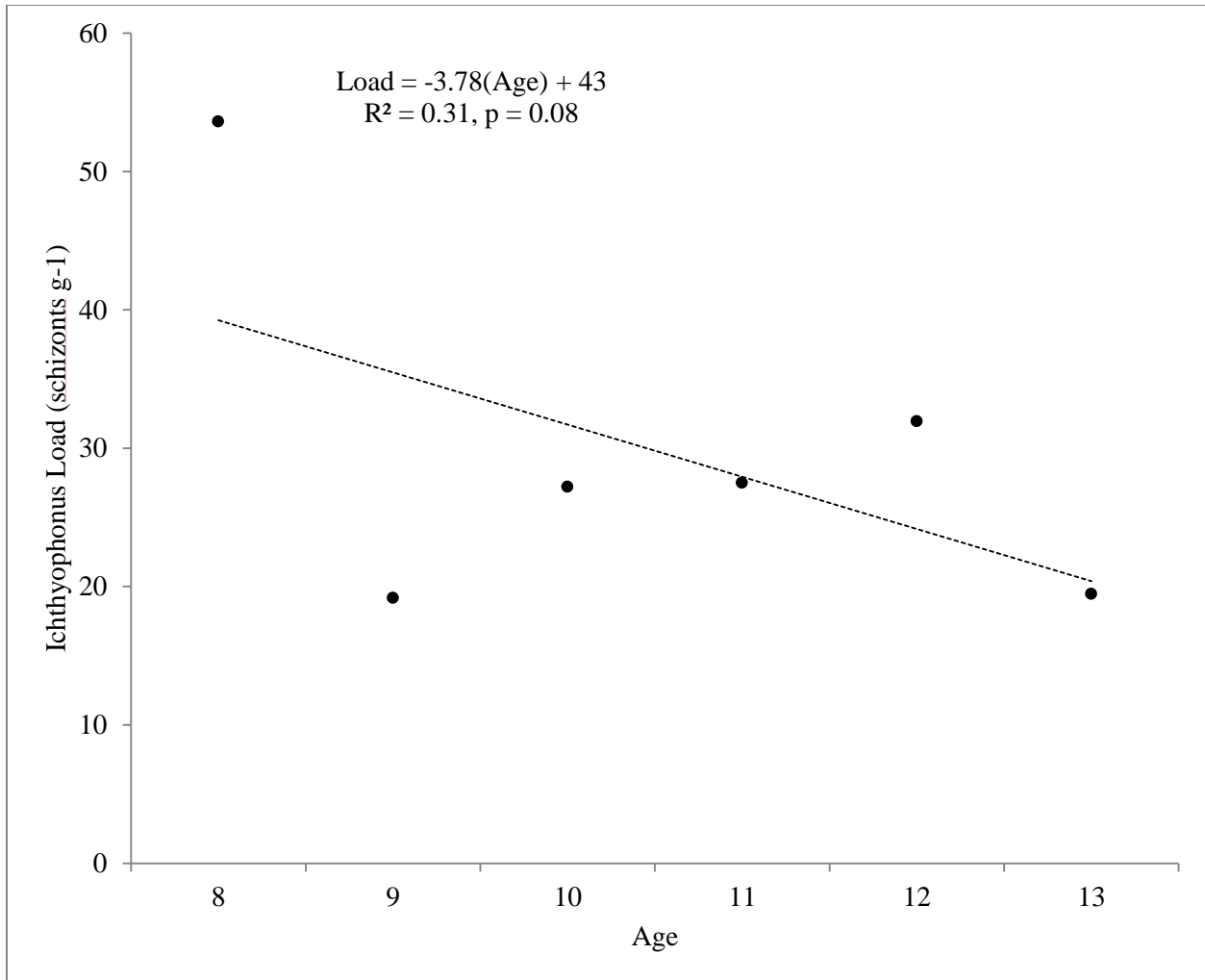
Figure 2.4 Average *Ichthyophonus* tissue burdens for age classes; data were truncated to exclude age classes with n < 7. When truncated age accounts for 31% of variability in *Ichthyophonus* load.

Total estimated schizonts per heart

Total schizonts were estimated for the entire heart by multiplying 0.5 (amount of heart taken for culture) by the specific intensity of that heart, then adding the number of schizonts observed. There was a wide range of total estimated schizonts among all samples, ranging from 6 to 1,245, with no correlation of total schizonts to age.

Parasite load range

Parasite load varied among both genders and within all ages. Females had a higher load, ranging from 2 to 100, while males ranged from, 3 to 62 (Figure 2.5).
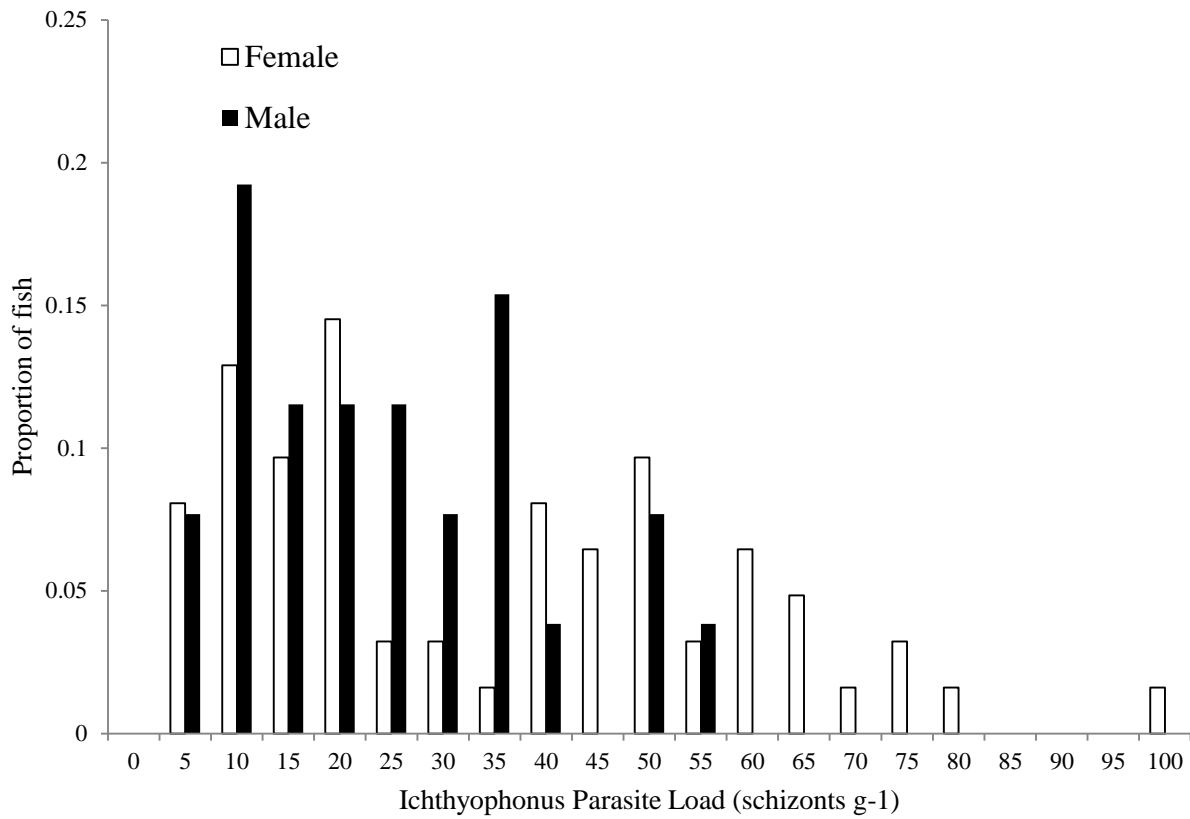
Figure 2.5 *Ichthyophonus* tissue burdens within bin sizes and the associated proportion of male and female fish. Female fish had a wider array of intensities.

2.4 DISCUSSION

Intensity varied greatly and was not correlated with gender or age (Figures 2.1 – 2.4). This result is not unexpected, however, due to the variability of individual reactions to pathogens. Potential confounding factors that could have affected these results include initial host exposure level, time since exposure, and host immune response. Kocan *et al.* (2013) determined that species and individuals react differently to the same exposure level. By examining tissue explants and aortic blood cultures within sculpin and rainbow trout, *Ichthyophonus* schizonts were found within both the aortic blood cultures and cardiac muscle as soon as 6 hours post exposure within sculpin and found in aortic blood cultures within 24 hours in rainbow trout and 96 hours for cardiac muscle. After 6 days post exposure, though, *Ichthyophonus* schizonts were only sporadically recovered from the blood of both species, suggesting that a transient parasite dissemination period occurs shortly after exposure. To determine a correlation between parasite load and host mortality we suggest laboratory testing to control for factors such as initial levels of host exposure and time since exposure. By controlling for these factors, parasite load and its effect on host growth, swimming capabilities, and mortality can be observed and potentially provide insight as to why some fish are affected by infection while others are not.

Kocan *et al* (2013) further determined that there are multiple stages of schizont growth, resulting in varying sizes, which was observed in our study as well. Schizonts circulate throughout the blood until they become too large to pass through the capillary beds and become lodged within organs, which in this study was the heart. One aspect which was not examined in this study is the difference in heart weight between infected and non-infected halibut. In a study examining the difference in swimming stamina among infected and non-infected rainbow trout, Kocan *et al.* (2006) found that infected hearts weighed 40% more due to parasite biomass and granulomas.

I suggest further research to include investigating different dyes or filtration systems. Only one stain was used, instead of H & E or PAS staining process, because the filtration paper became too saturated and schizonts were not easily identifiable. In addition to investigating different dyes we suggest laboratory testing to validate the pepsin digestion and morphology procedures. Two tests that would confirm the identity of the isolated *Ichthyophonus* schizonts are PCR and CISH. We also suggest separating the heart into multiple sections and digesting separately. This could help determine whether *Ichthyophonus* tends to occupy one section of the heart more than others, thereby increasing the likelihood of detection while using the culture or histology method.

When performing field surveillance for *Ichthyophonus* in populations of wild marine fishes, survey results are typically presented as total infection prevalence, with little mention of infection intensity or parasite load. By using pepsin digestion, individual schizonts were isolated and enumerated, therefore obtaining a quantifiable measure of parasite load in infected fish tissues. Although other studies used pepsin digestion to determine parasite presence and density, such as whirling disease in salmonids, there is no documentation of it being used to obtain parasite load within internal organs.

I believe that the pepsin digestion method could prove useful in a multitude of ways. Pepsin digestion could be a quick and affordable method for initial detection, and proves more effective than histology because the entire organ is being processed instead of small sections that are not guaranteed to contain *Ichthyophonus*. Kocan *et al.* (2011) encountered this uneven distribution in a study determining density within exterior ulcers and the associated underlying muscle, which showed a wide array of schizonts. By utilizing this method, not only can prevalence rates be obtained and applied at the population level, but parasite load rates can be established to

reveal potential physiological changes within individuals. With *Ichthyophonus* detection in new

fish species, particularly economically and ecologically important stocks, it is critical to

understand how this disease affects species and individuals differently.

GENERAL DISCUSSION

The primary objective of this study was to determine the prevalence of *Ichthyophonus hoferi* in Pacific halibut and investigate how factors, such as gender, length, and age affect that prevalence in such fish within Cook Inlet, Alaska, through the testing of sport caught fish sampled in the port of Homer. Over a two year span, 26% of Pacific halibut were infected with *Ichthyophonus*. Neither sex nor length impacted infection prevalence, with infected fish not significantly varying in size from uninfected fish. Age and prevalence, however, were positively correlated (y = 0.02(Age), R^2 = 0.31, P = 0.004). *Ichthyophonus* prevalence increased approximately 2% per year between the ages of 2 and 16, with 50% of 16 year old fish carrying *Ichthyophonus*. This finding is analogous to previous studies that establish that as fish increase in age, their probability of encountering disease or infectious agents also increase due to cumulative and recurrent exposures. Thus elevated *Ichthyophonus* prevalence rates are present in older cohorts (Hershberger *et al*. 2002). Age and size among Pacific halibut are also positively correlated. Interestingly, historical data obtained from the International Pacific Halibut Commission (IPHC) states that Pacific halibut size at age has decreased, although hypotheses are still being tested as to why (IPHC 2014).

The second objective of this study was to test a new method of *Ichthyophonus* detection, via pepsin digestion, its effectiveness to isolate and quantify *Ichthyophonus*, and the relationship between *Ichthyophonus* intensity, host sex, and age. Overall, sex did not have an effect on intensity. However, females had a higher range of intensities, from 2 to 100, while male intensity ranged from 3 to 62. Unlike prevalence, intensity was not correlated with age (y = -0.95(Age) + 40, R^2 = 0.03, P = 0.113). These results are not unexpected, however, due to the variability of how individuals react to pathogens. Potential confounding factors that could have

affected these results include the initial exposure level, time since exposure, and host immune response. The pepsin digestion method proved to be effective in isolating *Ichthyophonus* schizonts and could be employed for further research in obtaining prevalence rates, in addition to intensities. Although intensities were not affected by age or gender, prior research indicates that species respond to *Ichthyophonus* infection differently, and thus, this issue should be investigated further (Kocan *et al*. 2013).

We speculate that Pacific halibut become infected by eating infected prey. It is difficult, however, to determine potential transfer hosts for Pacific halibut as they are opportunist feeders (Blaylock *et al.* 1998). Pacific halibut's diet includes a wide array of prey including fish, crustaceans, cephalopods, marine worms, kelp, forage fishes, and fisheries offal. In an effort to identify potential transfer hosts, a study was conducted by Sarah Webster on stomach contents and isotope analysis of Pacific halibut prey in conjunction with *Ichthyophonus* sampling. Although analysis is ongoing, two fish species known to carry *Ichthyophonus*, Chinook salmon and Pacific herring*,* were found in sampled Pacific halibut stomachs and isotope analysis.

In a preliminary North Pacific Research Board (NPRB) study, *Ichthyophonus* was detected within Pacific halibut in the northern Bering Sea, Prince William Sound, and Oregon Coast at prevalence rates of 29.7%, 58.3%, and 23.7% respectively (IPHC RARA, project #1015, 2013) and most recently at 26% within the Cook Inlet, Alaska. This newest detection could possibly be attributed to increased awareness, recent introduction of *Ichthyophonus* to new hosts, or changing environmental conditions. Since it is established that piscivorous fish can become infected by eating infected tissue, I suggest a cease in the practice of grinding and discarding carcasses back into marine waters, creating a potential new source of infection. We also suggest continuing the monitoring program as very little is known about how *Ichthyophonus* affects

Pacific halibut and other economically and ecologically important Alaskan marine fishes. Also,

the *post-mortem pre-dumpster* method should be utilized within all major ports as it is both cost

and time effective.

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