Proof of Concept: Efficacy of cleaner fish, cultured juvenile cunner (*Tautogolabrus adspersus*), for sea lice (*Lepeophtheirus salmonis*) mitigation and control in Atlantic salmon (*Salmo salar*)

by

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ABSTRACT

A laboratory-based sea lice culture system was established for hatching and rearing *Lepeophtheirus salmonis* (from egg strings removed from farmed Atlantic salmon) to the copepodid stage of development, and an enumeration method for estimating the number of sea lice copepodids required for artificial sea lice parasitism was developed. Investigation of the delousing efficacy of cultured juvenile cunner against *L. salmonis* artificially infecting Atlantic salmon smolts under laboratory conditions indicated that there was no significant effect of cunner stocking density on mean sea lice numbers when held in cohabitation for seven days at stocking densities of 0, 4 and 10% (p=0.143) and they did not exhibit delousing activity. Fin condition (as an indicator of fish welfare) of Atlantic salmon smolts was evaluated during this period. There was no significant effect of cultured juvenile cunner stocking density on mean dorsal fin erosion scores (p=0.463) and mean caudal fin erosion scores (p=0.591) for Atlantic salmon. Additionally, there was no effect of high (18°C) and low (2°C) water temperature on the delousing efficacy of cultured juvenile cunner against *Lepeophtheirus salmonis* infecting Atlantic salmon smolts during a separate seven-day period of cohabitation (p=0.093), and no economically important pathogens or reportable diseases (within the Atlantic Canada region) (e.g., BKD, IPNv, ISAv, VHSv, IHNv and Nodavirus) were detected in either species during this time.

GENERAL SUMMARY

Sea lice are naturally occuring marine ectoparasites which affect wild and farmed Atlantic salmon. For the long-term sustainability of the Atlantic salmon farming industry it is crucial to explore various measures to manage sea lice. Cleaner fish, species of fish which remove sea lice from other fish, are considered an environmentally-friendly biological approach to sea lice management. Cunner, a local cleaner fish species found naturally in waters off Atlantic Canada, have been identified as a potential cleaner fish candidate. To verify the cunner's ability to remove sea lice from Atlantic salmon, cultured juvenile cunner were held with Atlantic salmon smolts, artificially infected with sea lice, at several stocking densities (0, 4 and 10%) and at two water temperatures (2 and 18°C) under laboratory conditions. The results of this research indicated that the cunner did not remove sea lice from Atlantic salmon at various stocking densities or water temperatures but that both cunner and salmon remained healthy when housed together for seven days.

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LIST OF ABBREVIATIONS

°C	Degree centigrade
g	Grams
kg	Kilograms
L	Liter
µm	Micrometer; Micron
mL	Milliliter
ppt	Parts per thousand
ASK	Atlantic Salmon Kidney
AVC	Atlantic Veterinary College
BA	Blood Agar
BC	British Columbia
BKD	Bacterial Kidney Disease
BMA	Bay Management Area
CAD	Canadian dollar
CASD	Centre for Aquaculture and Seafood Development
CHSE	Chinook Salmon Embryo
DFO	Department of Fisheries and Oceans Canada
EPC	Epithelioma Papulosum Cyprini
FAO	Food and Agriculture Organization
HWT	High water temperature
IFAT	Indirect fluorescent antibody testing

IPN	Infectious Pancreatic Necrosis
IPNv	Infectious Pancreatic Necrosis virus
ISA	Infectious Salmon Anaemia
ISAv	Infectious Salmon Anaemia virus
IHN	Infectious Haematopoietic Necrosis
IHNv	Infectious Haematopoietic Necrosis virus
JBARB	Dr. Joe Brown Aquatic Research Building
LWT	Low water temperature
MA	Marine Agar
MUN	Memorial University of Newfoundland
NB	New Brunswick
NL	Newfoundland and Labrador
NS	Nova Scotia
OSC	Ocean Sciences Center
PIT	Passive Integrated Transponder
RAS	Recirculating aquaculture system
RDC	Research & Development Corporation
RT-PCR	Reverse transcription polymerase chain reaction
SCUBA	Self-contained underwater breathing apparatus
SKDM	Selective Kidney Disease Medium
TSA	Trypticase Soy Agar
UPEI	University of Prince Edward Island

USA	United States of America
USD	United States dollar

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CO-AUTHORSHIP STATEMENT

The proof-of-concept research described in this thesis was conducted by Zhiyu Chen, with guidance and support from Dr. Jillian Westcott[a], Dr. Nicole O'Brien[b] and Cyr Couturier[a]. Zhiyu Chen was responsible for co-designing and executing the experiments for Chapters 2, 3 and 4, the data collection associated with each chapter, the data analysis associated with each chapter (with support from Dr. O'Brien), and writing the thesis (including Chapter 1) with support from Dr. Westcott. As members of Zhiyu's MSc supervisory committee, Dr. Nicole O'Brien and Cyr Couturier provided feedback related to experimental design, data analysis and thesis editing. While no manuscripts have been submitted to date, the intent is to submit Chapters 3 & 4 as a single paper with Zhiyu Chen as primary author, Dr. Jillian Westcott, Dr. Nicole O'Brien and Danny Boyce as co-authors.

Several others were integral to the completion of this research and thesis. For Chapter 2, Dr. O'Brien and Mr. Sean Hickey[c] coordinated the field collection of sea lice from Atlantic salmon farms. Mr. Danny Boyce[d] and Cooke Aquaculture provided the cultured juvenile cunner required for Chapters 3 and 4. Dr. Nicole O'Brien[e] coordinated the collection and analysis of fish samples and provided the interpretation of the disease surveillance reports described in Chapter 4. Several research and technical personnel from the Marine Institute's Centre for Aquaculture and Seafood Development (CASD), namely, Chris Dawe[a], Mark Santos[a], Tracy Granter[a], and Marsha Clarke[a], provided their assistance with samplings (e.g., sea lice counts) and data collection throughout the course of each experiment.

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CHAPTER 1. General Introduction

1.1 Atlantic salmon aquaculture

Between 1970-2010, global aquaculture production increased more than 20-fold from 2.6 to 60.4 million tonnes (Asche et al., 2013). In 2017, the Food and Agriculture Organization (FAO) indicated that 53.4 million tonnes of aquaculture production was supplied by finfish aquaculture, including both inland freshwater species (e.g., carps, tilapia, catfishes) (45.6 million tonnes) and marine species (e.g., salmonids, groupers) (7.8 million tonnes) (FAO, 2019). According to the most recent FAO yearbook on Fishery and Aquaculture Statistics (FAO, 2019), aquaculture production has steadily increased and is approaching wild capture levels, with marine finfish aquaculture accounting for 14.6% of total finfish aquaculture production by volume. Some marine finfish species are considered important trade commodities, in terms of value, compared to inland freshwater farmed species; this is due to the fact that most farmed marine species, particularly salmonids such as Atlantic salmon (*Salmo salar)* and rainbow/steelhead trout (*Oncorhynchus mykiss*), have a higher market value per unit than most inland finfish species (FAO, 2019).

Production in the global salmonid aquaculture sector increased from 12,000 tonnes to over 2.4 million tonnes from 1980 to 2011 with a market value exceeding 10 billion USD in 2011 (Asche & Bjorndal, 2011; Asche et al., 2013). The major commercially farmed salmonid species include Atlantic salmon (*Salmo salar*), rainbow/steelhead trout (*Oncorhynchus mykiss*), coho salmon (*Oncorhynchus kisutch*) and Chinook salmon (*Oncorhynchus tshawytscha)*. In the late 1990s, Atlantic salmon superseded steelhead trout in production volume and became the dominant species in

global salmonid aquaculture (FAO, 2016). In 2010, Atlantic salmon contributed 77.9% of total global salmonid aquaculture production, followed by rainbow/steelhead trout at a value of 15.2% (Asche et al., 2013). The total production volume of farmed Atlantic salmon has continued to increase and was estimated as 2.3 million tonnes in 2018 (MOWI, 2020). To date, the leading producer countries of Atlantic salmon are Norway, Chile, the United Kingdom (mainly Scotland), Canada and the Faroe Islands (Asche et al., 2013). Norway has dominated production throughout the history of global Atlantic salmon aquaculture (Asche & Bjorndal, 2011). Chile currently ranks second, except for the year 2010 when production was significantly reduced due to an outbreak of Infectious Salmon Anaemia (ISA) (Asche et al., 2009; Asche et al., 2013). Global Atlantic salmon production in 2010 was 1,446,200 tonnes, including Norway (944,600 tonnes, 65%); UK (mainly Scotland) (141,800 tonnes, 10%); Chile (129,500 tonnes, 9%); Canada (118,000 tonnes, 8%); the Faroe Islands (42,100 tonnes, 3%) and other minor producers (e.g., the USA, Australia, etc.) (70,200 tonnes, 5%) (Liabø et al., 2011, as cited by Torrissen et al., 2011).

Canada currently ranks fourth globally in Atlantic salmon production following Norway, Chile and Scotland. In Canada, British Columbia (BC) has the highest Atlantic salmon production (by volume), followed by New Brunswick (NB), Newfoundland and Labrador (NL) and Nova Scotia (NS) (Chopin, 2015; DFO, 2017). From 2016 to 2017, Canadian Atlantic salmon production surpassed 120,000 tonnes, with a value exceeding 1 billion CAD, and representing over 80% of Canadian finfish aquaculture production (by volume and value). British Columbia (BC) remained the industry leader, supplying

90,511 tonnes in 2016, 85,608 tonnes in 2017 and 88,834 tonnes in 2018 (DFO, 2019), which accounted for over 50% of Canadian Atlantic salmon aquaculture production. The remaining production was contributed by the Atlantic Provinces, including Newfoundland and Labrador (NL) (DFO, 2019). Currently in Newfoundland and Labrador, open-ocean salmonid aquaculture production occurs on the south coast of Newfoundland (DFO, 2019), where total salmonid production in 2018 was 17,978 tonnes (mainly Atlantic salmon with a limited amount of rainbow/steelhead trout being produced) (DFO, 2019).

1.2 Disease challenges in salmonid aquaculture

As is the case with other commercially farmed animals, pathogens (such as bacteria, viruses, fungi and parasites) have been documented globally in all Atlantic salmon producing countries (Pettersen et al., 2015). As commercial salmonid aquaculture production continues to expand, impacts on aquatic animal health due to pathogens remain a potential on-going fish health issue (Pike & Wadsworth, 1999; Johnson et al., 2004; Torrissen et al., 2013). Therefore, pathogen identification, through the development of aquatic animal health surveillance programs is essential. Further, Fish Health Management and Integrated Pest Management Plans to implement appropriate treatment and control strategies against potential disease outbreaks, are crucial elements to the economic viability and long-term sustainability of the Atlantic salmon aquaculture industry.

1.3 Sea lice

"Sea lice", a plural form of "sea louse", refers to a group of naturally occurring ectoparasitic copepods in the order Siphonostomatoida, family Caligidae. At present, 37 genera and approximately 559 species are commonly found on both wild and farmed marine finfish globally. Approximately 162 species are categorized in the genus *Lepeophtheirus* and 268 species in the genus *Caligus* (Ahyong et al., 2011). *Lepeophtheirus salmonis* and *Caligus elongatus* affect global salmonid aquaculture as they require significant attention for management and mitigation (Pike & Wadsworth, 1999; Igboeli et al., 2014).

Sea lice have been considered the most economically significant parasites in over a 40-year history of the global commercial salmonid aquaculture industry (Pike & Wadsworth, 1999; Bravo, 2003; Costello, 2009; Abolofia et al., 2017). Currently, sea lice can be found in all major commercial salmonid farming areas globally, but the occurrence of the particular species varies by geographic location and host specificity. *L. salmonis* is responsible for the majority of sea lice infestations in all commercial Atlantic salmon farming areas of the Northern Hemisphere, including Norway, Scotland and Canada (both Atlantic and Pacific coasts of Canada), and to a lesser extent, Ireland and the Faroe Islands. Impacts on the health of the animals due to the presence of of *L. salmonis* have been reported in Norway since the 1960's, Scotland since mid-1970, Ireland since the late 1980's, and the east coast of Canada since the 1990's (Pike & Wadsworth, 1999). *Caligus elongatus* occurs and affects all major commercial Atlantic salmon farming areas in the Atlantic Ocean (e.g., Norway, Scotland, Ireland, Faroe Islands and the Atlantic coast of

Canada). *Caligus clemensi* is found on farmed salmonids on the Pacific coast of Canada (Pike & Wadsworth, 1999; Johnson et al., 2004), while *Caligus rogercresseyi* and *Caligus teres* only occur in the Southern Hemisphere and have been mainly responsible for sea lice infestations in commercial Atlantic salmon farming areas in Chile since the 1980s (Bravo, 2003; Johnson et al., 2004).

L. salmonis is host-specific to wild and farmed salmonids of the genera *Salmo*, *Onchorhynchus* and *Salvelinus*. In comparison with rainbow/steelhead trout and coho salmon, Atlantic salmon (*Salmo salar*) was found to have the thinnest epidermal layer and least dense distribution of mucous cells (their first defense layer against sea lice infestation), making Atlantic salmon more susceptible to *L. salmonis* compared to other farmed salmonids species (Fast et al., 2002). Skern-Mauritzen et al. (2014) distinguished biological and genetic differences in *L. salmonis* in the Atlantic and Pacific oceans, suggesting that Atlantic *L. salmonis* and Pacific *L. salmonis* should be further categorized into allopatric subspecies: *Lepeophtheirus salmonis salmonis* and *L. salmonis oncorhynchi* subspecies novo, respectively. *L. salmonis* has been found on the three-spine stickleback in the waters of British Columbia, Canada (Jones & Prosperi-Porta, 2011), suggesting *L. salmonis* in the Pacific Ocean has a broader host range than previously described. Most *Caligus* species are not host-specific and can be found on both wild and farmed salmonids, and occasionally on other marine finfish (e.g., herring, three-spine stickleback) (Pike & Wadsworth, 1999; Johnson et al., 2004; Jones & Prosperi-Porta, 2011).

1.3.1 Life cycle of *Lepeophtheirus salmonis*

The life cycle of *L. salmonis* was previously documented as consisting of ten developmental stages: nauplius I and II (two stages), copepodid (one stage), chalimus I to IV (four stages), pre-adult I and II (two male/female stages) and adult (one male/female stage), with a moulting process between each developmental stage (Schram, 1993). Subsequently, it has been suggested that only two chalimus stages (chalimus I and II) exist, suggesting the former chalimus stages are not separated by a moulting process. Hamre et al. (2013) suggested combining the former chalimus I and II stages into a chalimus I, and the former chalimus III and IV stages into a chalimus II. Therefore, the updated life cycle of *L. salmonis* comprises eight developmental stages (Igboeli et al., 2014; Appendix A). This eight-stage life cycle can be further classified into two distinct phases: a free-swimming planktonic phase (i.e., nauplius I and II and copepodid) and a parasitic phase (i.e., chalimus I and II, pre-adult I and II and adult). The last three stages (i.e., pre-adult I and II and adult) are often referred to as "mobile" stages, as *L. salmonis* at these stages can move freely on the external surface of the host (Johnson & Albright, 1991).

Generation time of the *L. salmonis* life cycle varies with water temperature, salinity, photoperiod and light intensity under different environmental conditions (e.g., laboratory and open-ocean). The estimated average generation time of *L. salmonis* ranges from 6-8 weeks at 9-12 ºC in saltwater (salinity 30-33 ppt) (Johnson & Albright, 1991; Hamre et al., 2013). Subsequent to hatching from the egg (post-eclosion), *L. salmonis* larvae (nauplius I and II) are capable of free-swimming in saltwater by using three pairs

of anterior protrusions. The size of nauplius I and II is not significantly different, averaging 0.3-0.5 mm in length and 0.2-0.3 mm in width (Johnson & Albright, 1991; Schram, 1993). The body shape transforms from an oval shape (nauplius I) to an elongated shape (nauplius II). The body of *L. salmonis* nauplii appears mostly transparent but red-brownish pigments can be observed in different regions inside the body when viewed under a compound microscope (Schram, 1993). Nauplii are photosensitive and have a single pair of eyespots on the dorsal anterior end. Both stages survive on their yolk sacs, depending on their internal reserves for nutrients (Pike & Wadsworth, 1999).

As nauplii moult to copepodids, their body size develops to 0.7-0.8 mm in length and 0.3-0.4 mm in width. The copepodid stage represents a crucial transition period from the planktonic to the parasitic phase. Newly moulted copepodids are free-swimming and can remain planktonic for a short period of time as they seek out a host on which to attach. The capability of the free-swimming copepodids to detect, attach and settle on the host fish is influenced by water temperature, salinity, light intensity and hydrodynamic factors in their environment (Heuch, Parsons & Boxaspen, 1995; Pike & Wadsworth, 1999; Heuch, Nordhagen & Schram, 2000). Copepodids that fail to find a host will eventually die after they utilize the internal nutrients provided in their yolk sac. Following attachment on a host, most copepodids cluster and settle on fins and other areas which encounter slower water currents. Although the copepodids remain non-feeding for a while, they start to form a simple functional digestive system consisting of mouth, gut and anus (Pike & Wadsworth, 1999). An external structure referred to as the frontal filament, develops at the anterior end of the head area, which marks the end of the

copepodid stage and indicates the start of its development into the chalimus stage. The frontal filament facilitates the ability of the chalimus stage to penetrate through the fish scales to the epidermis, strengthening their attachment on host fish. At this point, chalimi obtain nutrients via their feeding behaviour on the epidermis, tissues and mucus of the host fish. Chalimi can grow up to 1.2-2.8 mm in length and 0.3-1.3 mm in width. Once they develop into chalimus II, they are visible with the naked eye. However, because they blend in with the dark skin color of their host, or shelter on the gills underneath the operculum of the host fish, they can be difficult to identify with the naked eye when present on the host fish (Schram, 1993; Pike & Wadsworth, 1999).

The development of genital segments of both male and female lice indicates sexual maturation of the pre-adult *L. salmonis* and mating between male and female pre-adults subsequently occurs. The gradual disappearance of the frontal filament enables pre-adults to move freely and become mobile on or between host fish. Pre-adults remain attached to their host by using their cephalothorax as a 'suction cup' (Schram, 1993; Pike & Wadsworth, 1999). They are commonly found clustered on the dorsal fin, head, and posterior ventral areas of the host fish, where the epidermis is thinner and there are less scales to protect the host fish from sea lice infestation (Pike & Wadsworth, 1999). The mating or copulating pairs, which consists of a pre-adult male attached to the genital segment of a pre-adult or adult female, start to appear during this period. Size differentiation of *L. salmonis* male and females is evident from the pre-adult stages to the adult stage. On average, *L. salmonis* adult females grow larger than adult males. Pre-adult males measure from 3.4-3.6 mm in length and 1.6-1.9 mm in width and adult males

measure 5-6 mm in length and 2 mm in width, while pre-adult females measure 3.6 mm in length and 1.9 mm in width and they can develop to adult females with a length of 8-11 mm (including egg strings) and a width of 3-5 mm (Schram, 1993; Revie et al., 2002). In addition, sexual dimorphism becomes apparent in relation to the shape of the genital segments as males form an "oval shape or barrel shape" and females a "W-shape or horseshoe-shape" (Schram, 1993). Overall, these changes can be employed to differentiate between *L. salmonis* sexes and stages during sea lice identification and counting (Wootten, Smith & Needham, 1982).

1.3.2 Pathological effects and economic impacts of sea lice infestation on Atlantic salmon aquaculture

Pathological effects of sea lice infestation have been investigated under laboratory and field conditions on farmed salmonids and other wild fish (Finstad et al., 2000). One of the most commonly observable pathological symptoms is physical damage on the external body surface of the infected host, such as body lesions and fin erosion, which are mainly caused by sea lice feeding behaviour on skin tissue, mucus and blood of the host (Pike & Wadsworth, 1999). The severity of the damage on the host's epidermis is dependent upon a number of variables related to the host-parasite interaction, including host susceptibility (Fast et al., 2002), the host's size, age, nutrition, immunocompetence, sea lice species and abundance (sea lice number and the corresponding developmental stages present on the host) (Tully & Nolan, 2002). Irritation caused by damage to the epidermis may result in the host exhibiting abnormal swimming patterns, such as leaping,

and twisting and turning (Wagner et al., 2003). If untreated, the sea lice will moult through mobile pre-adult and adult stages and feed more aggressively on the host's epidermis, mucus and blood. Laboratory experiments with heavy sea lice burdens (e.g., a range of 30-250 lice per fish) resulted in significant disruption of the physiological and immunological status of the host, causing elevation in a number of fish stress indicators (i.e., plasma cortisol, glucose, chloride levels), osmoregulatory and respiratory problems, and sudden mortality in some severe cases (Grimnes & Jakobsen, 1996; Bowers et al., 2000; Finstad et al., 2000; Wagner et al., 2003; Fast, 2014). Damage to the host's epidermal layer, the host's first defense barrier, as well as disruption and suppression of the internal physiological and immunological status, can leave the host more susceptible to opportunistic infections caused by both primary and secondary pathogens (Nylund et al., 1994; Jarp & Karlsen, 1997; Glover et al., 2006; Barker et al., 2019).

Farmed Atlantic salmon impacted by sea lice, may experience compromised feeding (e.g., food conversion efficiency and appetite) and growth during the production cycle, which significantly affects the profitability of the salmonid farming industry (Costello, 2009). A survey of Atlantic salmon farms in eastern Canada indicated that sea lice left untreated resulted in an estimated growth loss of 200 g per fish per production cycle on a site with approximately 200,000 farmed Atlantic salmon for the 2001 year production cycle, resulting in a growth loss of approximately 40,000 kg and a subsequent economic loss of 336,000 CAD (Mustafa et al., 2001). Rae (2002) estimated the economic losses associated with reduced growth caused by sea lice infestations accounted for at least 5% of the annual production value in Scottish Atlantic salmon

farms.

Costello (2009) described additional impacts of sea lice infestation on the profitability of salmonid farming. The overall expenses related to sea lice control (i.e., mainly chemical and biological treatments) ranked as the most significant cost. A comparison of data from all major Atlantic salmon farming countries (e.g., Norway, Chile, Scotland, Ireland and Canada), indicated that the cost of sea lice control averaged €0.19 per kg (or approximately 0.30 USD/kg). Costello (2009) used global salmonid production (approximately 1.6 million tonnes) in 2006 to determine that a total of €305 million (480 million USD) was spent globally on sea lice control by salmonid farming countries. Abolofia et al. (2017) quantified an economic cost of 436 million USD associated with sea lice infestations in 2011 for the Norwegian salmonid farming industry, which accounted for up to 9% of the annual salmonid production value that year. As noted by Costello (2009), variables affecting costs of chemotherapeutants for sea lice control involves purchase of chemotherapeutants, and the corresponding expenses associated with labour and equipment (e.g., well-boats, tarpaulins). Many other direct and indirect variables, such as the treatment type, timing, frequency, and efficacy of chemotherapeutants and equipment maintenance, increase the cost of sea lice control (Liu & Bjelland, 2014).

1.3.3 Sea lice control and Integrated Pest Management (IPM)

Various sea lice control methods and management strategies have been employed to control sea lice infestations in the Atlantic salmon farming industry since the 1970s

and these have been well documented and summarized (Costello, 1993; Roth & Sommervile, 1993; Pike & Wadsworth, 1999; Roth, 2000; Costello et al., 2001; Rae, 2002; Treasurer, 2002; Igboeli et al., 2014).

Integrated Pest Management (IPM) is a term that has been adopted by the global Atlantic salmon farming industry to describe a systematic approach toward sea lice management. The concept was originally developed for terrestrial agricultural pests (reviewed by Health Canada, 2003). The concept of IPM for sea lice management has been adopted by most salmonid farming countries and models of IPM have been devised and tailored to meet domestic situations and national jurisdictions (Pike, 2002). Integrated Pest Management has been defined in various ways. Two examples include, "…an overall management strategy that uses all available measures to suppress pests effectively, economically and in an environmentally sound manner" (Health Canada, 2003); and "…a multifactorial approach to pest management that involves a series of evaluations, decisions and controls that take advantage of all pest management options and strategies to achieve long-term solutions" (quoted in Roth, 2007). At present, sea lice control and management strategies are becoming more diverse, economical, innovative, and environmentally friendly due to ongoing efforts in scientific research and development.

1.3.4 Sea lice prevention strategies

Fallowing is considered an effective management strategy that is commonly used in commercial aquaculture practices for sea lice mitigation. Fallowing entails no

restocking of a new year-class of fish for a defined period of time post fish removal (Rae, 2002). Fallow periods can be variable, ranging from 4-6 weeks up to 1-2 years (Costello, 2006).

Single year-class separation means rearing a single-year-class of fish on one farm or several farms within one defined area at a time. In terms of farmed Atlantic salmon, newly stocked Atlantic salmon smolts in marine grow-out sites are more vulnerable to the saltwater environment and more susceptible to sea lice infestation in the first several months.

In addition to the stocking of the healthy fish, other preventive approaches, such as improving daily on-site fish husbandry practices, optimizing stocking density, and implementing regular monitoring for sea lice abundance on site, have been either applied or highly recommended for sea lice management in most Atlantic salmon farming countries, such as Norway (Costello, 1993), Scotland (Rae, 2002), Canada (Elmoslemany et al., 2013), Ireland (O'Donohoe et al., 2017) and Chile (Bravo, 2003).

1.3.5 Use of chemotherapeutants for sea lice control

Chemotherapeutants consist of bath and in-feed treatments. The application history, operation procedures and availability of chemotherapeutants associated with bath and in-feed treatments have been well documented, summarized and periodically updated in a number of comprehensive literature reviews spanning approximately three decades (Costello, 1993; O'Halloran & Hogans, 1996; Pike & Wadsworth, 1999; Roth, 2000; Grant, 2002; Burridge et al., 2010; Igboeli et al., 2014; Yossa & Dumas, 2016; Overton et

al., 2018). There are currently a number of chemotherapeutants available for controlling sea lice infestations (Treasurer, 2018; Poley et al., 2018). The compounds approved for use across the Atlantic salmon farming countries are variable due to the various regulatory frameworks and complex registration and licensing processes. For example, in Canada, chemotherapeutants administrated as bath treatments are considered pesticides/parasiticides and are regulated by Health Canada's Pest Management Regulatory Agency (PMRA), while those administered in-feed are considered drugs/medicines, and are regulated by Health Canada's Veterinary Drugs Directorate (VDD).

Chemotherapeutants used for sea lice control can be classified into five categories based on their chemical classes - organophosphates (azamethiphos); synthetic pyrethroids (cypermethrin and deltamethrin); oxidative agents (hydrogen peroxide); benzoylureas (chitin synthesis inhibitors: diflubenzuron, teflubenzuron, and lufenuron); and a semi-synthetic avermectin derivative (emamectin benzonate) (see Appendix B).

1.3.6 Sea lice resistance development to chemotherapeutants

In most Atlantic salmon farming countries, tolerance or resistance towards the chemotherapeutants have been reported (Denholm et al., 2002; Aaen et al., 2015; Overton et al., 2019). Tolerance development of sea lice (particularly *L. salmonis*) to azamethiphos, cypermethrin, deltamethrin, emamectin benzonate (Jones et al., 2013) and hydrogen peroxide (Treasurer, Wadsworth, & Grant, 2000) has been reported. However, resistance development to chitin synthesis inhibitors (i.e., diflubenzuron and

tefulbenzuron) has not been documented (reviewed by Aaen et al., 2015). Both the frequency of use and the administrated dosage of chemotherapeutants increased from 2005 to 2011 in Scottish salmon farms (Murray, 2015). Lack of rotation of chemotherapeutants with different modes of action, and reliance on a limited number of chemotherapeutants were all thought to contribute to resistance development (Denholm et al., 2002; Aaen et al., 2015). Therefore, the global Atlantic salmon farming industry is seeking sustainable and alternative mitigation strategies for sea lice control in order to avoid over-reliance on chemotherapeutants.

Innovative approaches and technologies have been developed and employed to support IPM for sea lice control. These include, but are not limited to, thermal treatment of fish with warm or cold water (e.g., Thermolicer® or Hydrolicer®) (Steinsvik, n.d.), use of filter-feeding bivalves which have been demonstrated to ingest planktonic sea lice when placed adjacent to fish farms (Bartsch et al., 2013), optical delousing technology (e.g., Stingray®) (Lekang et al., 2016, Stingray Marine Solutions (n.d.)), and a proposed long-term selective breeding program for Atlantic salmon (Gharbi et al., 2015). These innovative approaches and technologies broaden the range of options for sea lice control in Atlantic salmon aquaculture, although some of them have only been investigated in preliminary laboratory experiments or field trials. In order to prove their long-term efficacy and commercial feasibility for use in intensive commercial scale farming, more research and development, including field trials, need to be conducted to assist in an evidence-based approach to sea lice management.

1.4 Cleaner fish as a biological sea lice control

The use of cleaner fish species as a biological treatment against sea lice infestation has recently re-emerged in Norway and Scotland (Skiftesvik et al., 2013; Imsland et al., 2014; Skiftsvik et al., 2015) due to increasing evidence and concerns of sea lice (*L. salmonis*) resistance towards chemotherapeutants (Aaen et al., 2015; Helgesen et al., 2015), along with the rising attention dedicated to developing IPM strategies for sea lice control (Treasurer, 2002; Treasurer, 2018). Advantages of using cleaner fish for sea lice control include continuous efficacy for the duration of time that the cleaner fish are cohabitated with the Atlantic salmon, and less stress of handling and crowding of fish as is required during bath treatments (reviewed by Treasurer, 2002). However, the use of cleaner fish has challenges, such as technical problems (e.g., shipment and deployment of cleaner fish), ethical concerns (e.g., retrevial and reuse of cleaner fish post-treatment), biosecurity concerns (e.g., use of wild-caught cleaner fish without knowledge of their health record) and fish welfare and health (e.g., salmon pathogens affecting cleaner fish ,and vice versa) (Sayer, Treasurer & Costello, 1996; Treasurer, 2002; Treasurer, 2018).

1.4.1 History of the use of cleaner fish for sea lice control worldwide

The history of cleaner fish use in Atlantic salmon aquaculture dates back to the late 1980's in Norway. The idea was inspired by the natural symbiosis and cleaning behaviour demonstrated by some cleaner fish species found naturally in tropical waters. Åsmund Bjordal, a Norwegian fish biologist, documented the ability of several wild-

caught wrasses species (i.e., goldsinny, rock cook and cuckoo wrasse) to remove sea lice (*L. salmonis and C. elongatus)* from Atlantic salmon in laboratory experiments and subsequently in field trials between 1987 to 1989 (Bjordal, 1988; 1990; 1991). The Atlantic salmon farming industry in Europe took interest in the use of these cleaner fish candidates as a means by which to combat sea lice. Meanwhile, more evidence of the delousing ability of various cleaner fish species (e.g., goldsinny, rock cook, corkwing, ballan and cuckoo wrasse) was verified from laboratory experiments and field trials in Norway (Kvenseth, 1993), Scotland (Treasurer, 2002; 2005), and Ireland (Deady et al., 1995; Tully et al., 1996). Since all wrasse species were captured from wild wrasse fisheries in the 1990's, there was variability with respect to the number, species, and gender of wrasse captured. For example, in Norway, populations of wrasse used for sea lice control in the early 1990's were comprised of approximately 90% goldsinny wrasse and 10% mixed stock of rock cook and/or corkwing; ballan wrasse were not stocked during that time (Kvenseth, 1993). Knowledge regarding wrasse biology and wild capture techniques expanded due to the development of fundamental biological research during this period (Sayer et al., 1996). Although the use of wrasse for sea lice control expanded to several Atlantic salmon farming countries in Europe in the 1990s (e.g., Norway, Scotland), they were used in rotation with chemotherapeutants, which remained commonly employed. However, the attractiveness of using cleaner fish gradually diminished as new chemotherapeutants (e.g., emamectin benzoate, azamethiphos and cypermethrin) were introduced in the late 1990's, and there were reported challenges with cleaner fish use. These challenges were related to fish health (e.g., cleaner fish becoming

infected with furunculosis and vibriosis), concerns regarding wrasse as potential vectors for transmitting diseases to Atlantic salmon during cohabitation, and loss of wrasse due to mortality and escapement in sea cages over the winter months (Sayer et al., 1996; Treasurer, 2002; Treasurer, 2018). From 2000 to 2008, although the use of cleaner fish (wrasse) was not discontinued, the scale of their use remained limited, serving mainly as an alternative for, or in conjuction with, the strategic rotation of chemotherapeutants (Sayer et al., 1996; Treasurer, 2018).

The re-emergence of the use of cleaner fish for sea lice control occurred in Norway and Scotland around 2008. The commercial culturing of wrasse (e.g., ballan wrasse) provided another source of cleaner fish for the Atlantic salmon farming industry, along with wild-caught wrasse. Between 2013-2014, lumpfish (*Cyclopterus lumpus*) were identified as a cold-water cleaner fish species (Imsland et al., 2014). The commercial farming of ballan wrasse and lumpfish quickly expanded in scale and production, which subsequently supplied the demand required by the Atlantic salmon aquaculture industry for cleaner fish, thereby reducing pressure on wild wrasse fisheries (Powell et al., 2018). Currently, the use of wild-caught and cultured cleaner fish has been identified as a biological control method for sea lice control and a component of IPM strategies, in addition to the strategic use of chemotherapeutants (Treasurer, 2018). However, Overton et al. (2020) recommd that a more targeted evidence-based investigation is needed in terms of the extent of use of cleaner fish by the industry.

1.4.2 Cunner (*Tautogolabrus adspersus*) – a cleaner fish candidate in Atlantic Canada

In the early 1990's, following indication of positive outcomes associated with using several wrasse species for sea lice control in Europe, Canadian scientists and Atlantic salmon farmers began a search for native fish species that could serve as potential cleaner fish candidates (Levitan, 1991). In eastern Canada, MacKinnon (1995) initiated preliminary studies to verify the potential cleaning behaviour of wild-caught cunner (*Tautogolabrus adspersus*) for removing sea lice (*Caligus elongatus*) from farmed Atlantic salmon. In preliminary laboratory experiments, the total number of *C. elongatus* on Atlantic salmon smolts (18-22 cm in length, artificially infected with approximately 50 adult *C. elongatus,* held in 30-gallon glass tanks) decreased when held in cohabitation for 15 minutes with one wild-caught cunner (>10 cm in length). However, subsequent field trials conducted in sea cages in New Brunswick, Canada, where both species were stocked at a ratio of 30 wild-caught cunner to 2000 Atlantic salmon smolts, indicated that wild-caught cunner were ineffective at reducing numbers of *C. elongatus* during a 12 week period of cohabitation.

Published evidence with respect to the potential of cunner as a cleaner fish species for sea lice control has been limited to date. In early 2010, a preliminary experimental trial (e.g., placement of two cunner in a holding container with seawater and 40 sea lice) carried out at the Huntsman Marine Sciences Centre (HMSC) in New Brunswick, Canada, documented the interest of wild-caught cunner in consuming *L. salmonis* via visual observation and videotaping (DFO, 2014; Jones, 2015). Another experimental trial carried out in the same laboratory concluded that no irritation or aversion signs were

observed in cultured Atlantic salmon (no size indicated) when they were held with wild-caught cunners (length of cohabitation time and size of cunners not indicated), suggesting wild-caught cunner can be cohabited with cultured Atlantic salmon in tank environments .

In order to determine whether wild-caught cunner were able to actively remove *L. salmonis* from Atlantic salmon under field conditions, a trial was carried out at a commercial Atlantic salmon sea cage site in New Brunswick, Canada, whereby four stocking ratios (3, 6, 9 and 12% cunner) were employed in order to determine which ratio would achieve the highest reduction of *L. salmonis* on farmed Atlantic salmon (approximately 1.6 kg per fish) (DFO, 2014). The number of *L. salmonis* on Atlantic salmon were counted 24, 48, and 72 hours post-addition of wild-caught cunner (cunner size not indicated). Conclusions were made suggesting that wild-caught cunner were capable of removing *L. salmonis*, especially gravid females (adult females with egg strings), which was thought to be the most likely sea lice developmental stage consumed by wild-caught cunner. The field trials failed to determine an optimal stocking ratio due to the high variability of the sea lice count data. However, the stocking ratios of 9% and 12% cunner were not recommended because it was purported that, in the absence of a large scale wild cunner fishery, it is not economically viable and sustainable to use wild-caught cunner.

In Newfoundland and Labrador, Canada, Costa et al. (2016) investigated the delousing efficiency of wild-caught cunner (captured in the waters off Newfoundland) in tanks cohabited with cultured Atlantic salmon smolts artificially infected with adult *L.*

salmonis. A total of 150 passive integrated transponder (PIT) tagged farmed Atlantic smolts (averaging 210 mm in length and 148 g in weight) were housed in three experimental tanks (1364 L, 50 smolts per tank), with each tank of fish exposed (i.e., Atlantic salmon placed in holding containers with adult sea lice) to 100 adult *L. salmonis* (20 lice per smolt) prior to the addition of cunner. A stocking density of 10% cunner (5 cunners: 50 Atlantic salmon smolts) was employed in two of the experimental tanks with the third tank acting as the control (no cunner). Throughout the 78 hour trial period (3 days and a morning) following the addition of cunner to the tanks, continuous video recording under full light conditions above the surface of the water occurred for an average of 12 hours per day during daylight periods. Individual sea lice counts were conducted on all 150 smolts prior to the addition of cunner and 78 hour post-addition of cunner. In addition to sea lice counts, cunner behaviour was also documented (e.g., cunner chasing *L. salmonis* artificially infected Atlantic salmon smolts and several attempts of cunner picking sea lice off Atlantic salmon). Despite these observations, which the authors suggested demonstrated delousing behaviour, no significant reduction in *L. salmonis* numbers was detected in either of the experimental tanks containing cunner (10% cunner; 5 cunner: 50 Atlantic salmon smolts) compared to the control tank (no cunner) (p=0.275). The authors suggested that an optimal stocking ratio of cunner for optimal delousing efficiency requires further investigation (Costa et al., 2016).

While published information is available for cultured lumpfish as a cleaner fish species (Brooker et al., 2018), there is limited published and anecdotal information available related to wild cunner as a potential cleaner fish species, and to the author's

knowledge, there is no published information available with respect to the delousing ability of cultured cunner; a gap which the current research aimed to fill. However, a cleaner fish breeding program was subsequently initiated in research facilities in Newfoundland and Labrador and New Brunswick for cunner and lumpfish, respectively. The breeding programs were anticipated to expand fundamental knowledge in relation to wild-caught cunner broodstock management, spawning, egg incubation, larval and juvenile rearing under hatchery conditions, and to facilitate the development of a future source of commercial cleaner fish for the Atlantic salmon farming industry in Atlantic Canada (Boyce et al., 2018). The cultured juvenile cunner used in the current research were progeny of the cunner breeding program that was established at the Ocean Sciences Centre, Memorial University of Newfoundland (Boyce et al., 2018).

1.4.3 Biology of wild cunner (*Tautogolabrus adspersus*)

Cunner (*Tautogolabrus adspersus*) belong to the family Labridae and are related to the wrasse species which have been used as cleaner fish in Atlantic salmon aquaculture for sea lice control in Europe (Leclercq et al., 2014; Skiftesvik et al., 2014; 2015). Cunner are found to inhabit shallow and inshore marine environments in the waters of the western Atlantic Ocean, ranging from Newfoundland and Labrador and the Gulf of St. Lawrence in Canada to Chesapeake Bay in the United States (Johansen, 1925; Scott & Scott, 1988; FishBase, n.d.). In the waters off Newfoundland, cunner have been found to prefer shallow waters not below 8 meters in depth (Green & Farwell, 1971); similar observations showed that they inhabit shallow inshore reefs, rocky substrates, wrecks and

wharves, which they use as shelters to protect themselves from predators and winter conditions (Pottle & Green, 1979).

Similar to other members of the Labridae family, cunner have a wide range of skin coloration, including brown, grey, blue and green; this coloration is thought to function in mate attraction and the provision of a disguise for self-protection (Johansen, 1925; Green & Farwell, 1971). Maturity is thought to occur at 8-11 cm in length and their lifespan is up to 10 years where they reach a size of approximately 43 cm in length (Scott & Scott, 1988; MacKinnon, 1995).

Cunner have been described as a carnivorous species, with a wide range of food sources in the wild, and feeding preferences that vary by life stage. In the wild, juvenile cunner forage for planktonic crustaceans, and their feeding preference towards mussels, crabs and barnacles changes when they become adults (Chao, 1973). Wild juvenile cunner also feed on molluscs and microcrustaceans (e.g., copepods, amphipods, isopods, etc.) and larger cunner can consume more diverse food sources, including mussels, crabs, sea urchins, and to a lesser extent, marine worms, fish eggs and eelgrass plants (Scott & Scott, 1988). In the waters off Newfoundland, Green & Farwell (1971) observed that swimming behaviour and appetite of wild cunner diminished as water temperature decreased, becoming torpid and ceasing to feed below a water temperature of 5°C. It is hypothesized that their potential delousing ability is correlated with water temperature.

1.5 Cleaner fish health and welfare

Cleaner fish health and welfare was not of primary concern in the 1990s when

wrasse were first employed for sea lice management on Atlantic salmon farms in Europe, as the focus at that time was on the delousing efficacy and survival of wild-caught wrasse. In recent years, the challenges and problems associated with cleaner fish health and welfare have been highlighted as important factors for consideration when using wild-caught and cultured cleaner fish for sea lice management (Treasurer, 2012; Treasurer, 2018). As a component of IPM for sea lice control, the use of cleaner fish requires the maintenance of optimal fish health and welfare of both Atlantic salmon and cleaner fish when held in cohabitation. The evaluation of the fish health and welfare of cleaner fish and Atlantic salmon in cohabitation is multifaceted and requires further investigation.

1.5.1 Fin condition as an indicator of fish welfare

Various fin condition indices have been developed as a measure of fish welfare in numerous finfish species (Kindschi, 1987; Latremouille, 2003; Ellis et al., 2008). Among them, fin erosion was highlighted as a quantifiable parameter that can be used as a measure of fish welfare. Reasons associated with the occurrence of fin erosion in aquaculture species include overcrowded rearing densities, intraspecific aggression or competition between fish for space and food, rough or unhygienic rearing environments, pathogen infections (e.g., bacteria, virus, parasites or fungi) and nutritional deficiencies (Latremouille, 2003). For example, through laboratory observations related to fish behaviour, Turnbull et al. (1998) found that dorsal and caudal fin erosion of Atlantic salmon parr was likely attributable to intraspecific aggression when cohabitated in

commercial farms. Many fin condition indices have been developed for farmed salmonid species (e.g., Atlantic salmon and rainbow trout). One of the most commonly used fin erosion indices is a fin erosion scoring scale which uses different levels (e.g., 0-4 point scale) of fin erosion in conjunction with written descriptions or photographic keys. With the aid of various descriptive or scoring scales, fin condition (e.g., fin erosion, fin splitting) of various fish species (e.g., Atlantic salmon, rainbow trout, sea bream and sea bass) has been evaluated (Bosakowski & Wagner, 1994; MacLean, 2000; Hoyle et al., 2007; Person-Le Ruyet et al., 2007; Noble et al., 2008; Arechavala-Lopez et al., 2013). However, due to the fact there is no universally accepted fin erosion scoring scale, the scales employed are variable, making comparisons between studies difficult.

The assessment of fin condition (e.g., fin erosion and fin splitting) has been conducted on five wild-caught wrasse species (e.g., goldsinny, rock cook, corkwing, cuckoo and ballan wrasse) in field trials on Scottish Atlantic salmon farms, suggesting these indices may be an appropriate means by which to assess wrasse fin condition (Treasurer & Feledi, 2014). In a sea cage trial, Skiftesvik et al. (2013) found that ballan wrasse (wild-caught and cultured) and wild-caught corkwing wrasse caused fin bites and skin or gill damage to Atlantic salmon while removing sea lice (*L. salmonis*). Their results also suggested that corkwing wrasse might be more aggressive than ballan wrasse, causing greater damage to Atlantic salmon during cohabitation in sea cages. Leclercq et al. (2014) reported anecdotal observations of physical damage to the eyes, fins and skin of Atlantic salmon that may have been associated with cohabitation with cultured ballan wrasse in a laboratory tank trial. Based on these initial findings, it is reasonable to

surmise that potential fin erosion may be associated with some inter-species interactions (e.g., aggression) between cleaner fish species and Atlantic salmon during cohabitation.

To the author's knowledge, there has been no quantitative fin erosion assessment conducted on cultured Atlantic salmon held in cohabitation with wild-caught or cultured cunner. An investigation of the fin erosion condition of Atlantic salmon smolts cohabitated with cultured juvenile cunner could have implications on the selection of cleaner fish welfare indices and future application of this species for sea lice control.

1.5.2 Cleaner fish health

The prevalence of various bacterial and viral pathogens have been well documented in various cleaner fish species (e.g., wrasse and lumpfish) (Costello, 1993; Treasurer, 2012; Powell et al., 2018; Scholz, Glosvik & Marcos-López, 2018). Early research focussed on bacterial and viral diseases of wild-caught wrasse in Europe. The occurrence of disease in cleaner fish populations during the rearing process, and later during deployment in Atlantic salmon sea cages, is possible.

There have been no published reports of bacterial and viral diseases in cultured cunner in Canada, however, the practice of routinely screening cultured cunner for pathogens and the ongoing assessment of their health condition currently comprises an important component of disease surveillance programs in Atlantic Canada. With the aid of disease surveillance programs, the possibility of transmission of pathogens from cultured cunner to Atlantic salmon can be routinely monitored.

1.5.2.1 Bacterial and parasitic diseases and pathogens in cleaner fish

Bacterial diseases have resulted in many mortalities in cleaner fish when stocked in salmon sea cages and during the rearing process in commercial hatcheries (Scholz, Glosvik & Marcos-López, 2018). Typical and atypical furunculosis, a bacterial disease cause by *Aeromonas salmonicida*, has been detected and reported in wild-caught wrasse (e.g., goldsinny) in Norway, Scotland and Ireland (Treasurer, 2012). In addition, wrasse (e.g., goldsinny, corkwing) have been found to be susceptible to experimental infection with vibrio spp. (e.g., *Vibrio anguillarum*, *Vibrio splendidus*, *Vibrio tapetis*) under laboratory conditions (Jensen et al., 2003, reviewed by Treasurer, 2012). There have also been reports of bacterial diseases in wild-caught and cultured lumpfish, the most recently identified cleaner fish species in Europe. For example, the occurence of pasteurellosis in lumpfish has been documented in Norweigian culture facilities (Alarcón et al., 2016a). Additionally, a range of pathogens have also been detected in farmed and cultured lumpfish, namely *Nucleospora cyclopteri* (Microsporidia), *Kudoa islandica* (Myxozoa), *Tetramicra brevifilum* and *Tenacibaculum maritimum*, (Alarcón et al., 2016b; Scholz et al., 2017; Småge et al., 2016) and *Myxobolus albi* (Myxozoa) in wild captive lumpfish (Cavin et al., 2012).

1.5.2.2 Viral diseases in cleaner fish

As reviewed by Treasurer (2012), goldsinny wrasse have been found to be susceptible to Infectious Pancreatic Necrosis (IPN) under experimental conditions,

although this may not be the case under field conditions. The virus is not transmitted from wrasse to Atlantic salmon in cohabitation, although goldsinny wrasse are postulated to be a reservoir of IPN infection (Gibson, Smail & Sommerville, 1998). As documented in the review by Powell et al. (2018), viral haemorrhagic septicaemia (VHS) has been detected in farmed lumpfish in Europe. For example, Guðmundsdóttir et al. (2019) found that an outbreak of viral haemorrhagic septicaemia (VHS) in a lumpfish culture facility in Iceland was caused by a novel viral haemorrhagic septicaemia virus (VHSV) of genotype IV. New viral diseases of the family *Flaviviridae* have also been documented in farmed lumpfish (Skoge et al., 2018).

1.6 Current problem and research objectives

A cultured cunner population was established at the Ocean Sciences Centre (OSC) of Memorial University of Newfoundland to provide the salmonid aquaculture industry in Newfoundland with a predictable and sustainable source of cleaner fish (Boyce et al., 2018). While the delousing ability of wild-caught cunner has been documented in eastern Canada (DFO, 2014; Costa et al., 2016), the proclivity for cultured juvenile cunner to remove sea lice from Atlantic salmon when held in cohabitation is unknown, despite the fact that they are progeny of wild-caught broodstock. If cultured cunner exhibit a delousing ability under controlled laboratory conditions, the influence of key factors (e.g., stocking density and water temperature) on their delousing efficacy requires investigation.

The cohabitation of Atlantic salmon and cultured cunner may have implications

for potential inter-species interactions (e.g., aggression toward Atlantic salmon or fin damage caused by delousing activity of cultured cunner) and fish health. An evaluation of fin condition (e.g., fin erosion score) is a commonly employed indicator of fish welfare that was employed in the current study. The prevalence of pathogens in cohabitated fish species may present an obstacle to the success of using cultured cunner in Atlantic salmon sea cages for sea lice control. There is a paucity of information related to the susceptibility of cultured cunner and Atlantic salmon to disease-causing pathogens when they are cohabitated and the influence of both species (i.e., Atlantic salmon and cultured cunner) regarding potential inter-species disease transmission, when held in cohabitation under different water temperatures.

1.6.1 Research objectives

The overall objectives of this research were:

(1) To establish a sea lice culture system for hatching and rearing *L. salmonis* (from egg strings removed from Atlantic salmon reared in net pens) to the copepodid stage of development under laboratory conditions (Chapter 2);

(2) The development of an enumeration method for estimating the number of sea lice copepodids required for artificial sea lice parasitism (Chapter 2);

(3) To investigate the effect of stocking density of cultured juvenile cunner on their delousing efficacy against *Lepeophtheirus salmonis* artificially infecting Atlantic salmon smolts (Chapter 3);

(4) To evaluate fin condition (e.g., fin erosion score) of Atlantic salmon smolts when

cohabited with cultured juvenile cunner (Chapter 3);

(5) To investigate the effect of water temperature on delousing efficacy of cultured juvenile cunner against *Lepeophtheirus salmonis* artificially infecting Atlantic salmon smolts (Chapter 4);

(6) To examine the prevalence of several economically important pathogens or Reportable Diseases (within the Atlantic Canada region) (e.g., BKD, IPNv, ISAv, VHSv, IHNv and Nodavirus) in Atlantic salmon smolts and cultured juvenile cunner held in cohabitation (Chapter 4).

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CHAPTER 2. Establishment of sea lice (*Lepeophtheirus salmonis*) culture systems and laboratory sea lice cultivation and enumeration for artificial sea lice parasitism

2.1 Abstract

A laboratory reared population of sea lice (*Lepeophtheirus salmonis*) copepodids were developed for the artificial parasitism of Atlantic salmon (*Salmo salar*) smolts required in Experiments 1 (see Chapter 3) and 2 (see Chapter 4). This requirement necessitated the development of a sea lice culture system to cultivate field-collected *L. salmonis* egg strings to parasitic copepodids. For both separate experiments, a designated number of *L. salmonis* copepodids were added to tanks containing Atlantic salmon smolts and subsequently grown to mobile stages (i.e., pre-adult and adult *L. salmonis*) on the hosts prior to the addition of cultured juvenile cunners. Sea lice enumeration procedures were necessary to quantify the approximate number of copepodids required for the artificial sea lice parasitism of Atlantic salmon smolts. Various models of artificial sea lice parasitism have been applied in previous research, however, no standardized methods exist with respect to designing a sea lice culture system and procedures for culturing sea lice under laboratory conditions. Furthermore, information regarding the enumeration of infective copepodids for artificial sea lice challenges is limited. The objectives of this non-experimental chapter were to demonstrate a method for the design and establishment of a sea lice culture system and procedures for the cultivation and enumeration of *L. salmonis* copepodids under laboratory conditions. This chapter was not intended to investigate the effect of water temperature or other parameters on the hatching success of

sea lice egg strings to copepodid stages. The basic components required for establishing a laboratory sea lice culture system were determined following a review of scientific literature and consultation with experts from the Atlantic Veterinary College (AVC), University of Prince Edward Island (UPEI). Approximately 8-10 weeks prior to the commencement of Experiments 1 and 2, sea lice (female *L. salmonis* with egg strings) were collected from commercial Atlantic salmon farms in the Coast of Bays region of Newfoundland and Labrador. In the laboratory, *L. salmonis* egg strings were hatched and cultivated in the sea lice culture system until they developed to the infective copepodid stage. Their post-hatch developmental stages were categorized as Nauplius I, Nauplius II and Copepodid. As individual variations occurred in the time of hatching and further development of *L. salmonis* egg strings, a crude estimation of the percentage of *L. salmonis* at each developmental stage within each sea lice culture unit was recorded. A method was developed to enumerate *L. salmonis* copepodids for use in subsequent artificial sea lice parasitism for Experiments 1 and 2 (in Chapters 3 and 4).

2.2 Introduction

Under laboratory conditions, development of *L. salmonis* has been documented as occurring from 5 to 22°C. Adult male and female *L. salmonis* can grow up to an approximate length of 5-6 mm and 8-11 mm, respectively, grazing on skin, mucus and

blood of salmonid hosts (Johnson & Albright, 1991). The life-cycle of *L. salmonis* iscomprised of eight developmental stages (Hamre et al., 2013). Newly extruded fertilized egg strings have been recorded to develop to the adult stages in approximately 40 to 52 days at 10°C under laboratory conditions (Johnson & Albright, 1991). Each female *L. salmonis* has the potential to produce up to 11 pairs of egg strings at 7.2°C from a single mating and can live up to 191 days (Heuch et al., 2000). The number of eggs contained in individual pairs of egg strings ranges from 107 to 1220 (Hamre et al., 2009). Individual variation in egg strings, such as egg string length and percentage of viable eggs (fertilized eggs that are alive and can potentially hatch) has been documented and may be attributed to water temperature, salinity and egg string origin (Costello, 1993; Heuch et al., 2000). Boxaspen & Næss (2000) hatched individual pairs of egg strings and cultured them to the copepodid stage at five temperatures (2, 3, 4, 5 and 10°C) in separate incubation units, and determined that *L. salmonis* development time shortened as rearing water temperature increased; at 10°C, egg string hatching was first observed within 8.7 ± 0.1 days of the addition of the egg strings to the incubation units, and subsequent development to copepodids occurred up to 12.7 days later.

The attachment and subsequent survival and development of *L. salmonis* copepodids on salmonid hosts is crucial for the outcomes of artificial sea lice parasitism in laboratory experiments. Two key factors that impact upon sea lice attachment and

survival are water temperature and salinity (Tucker et al., 2000). For example, Tucker et al. (2000) found that, at 10 days post artificial parasitism on Atlantic salmon smolts (120 ± 6.18 g), a higher settlement, survival rate, and a faster further development of infective copepodids occurred at 12°C compared to 7°C in 34 ppt seawater. Their second experiment indicated that *L. salmonis* copepodids had a higher settlement and survival rate and faster development on Atlantic salmon smolts in 34 ppt seawater compared to 24 ppt seawater at the fixed water temperature.

Published information regarding the materials required and specifications for the design and operation of various sea lice culture systems, cultivation procedures and enumeration of infective copepodids, is limited. Early sea lice culture system designs consisted of a chamber (5 × 5 L) with 125 μm mesh for incubating egg strings, which was suspended in a flow-through seawater tank (34-35 ppt) at 9-10°C, in which the first observation of nauplius hatching from egg strings was recorded 9 days post-addition of egg strings to the culture chamber (Grimnes & Jakobsen, 1996). In 2000, Norwegian scientists tested several types of containers, including 250 mL plastic bottles (bottom removed, placed upside down) with aeration supplied (two aquarium air stones), 150 mL flasks, 30 mL Petri dishes and 10 mL multiwell dishes, for culturing individual pairs of egg strings at 5 or 10°C (Boxaspen & Næss, 2000). During that same time, Canadian scientists used 20 L white plastic buckets as a major component for establishing their sea

lice culture systems to cultivate sea lice (*L. salmonis*) in aerated seawater (27 ppt) under a photoperiod of 12hr light: 12hr darkness (Mustafa et al., 2000). To maintain the water temperature inside the buckets at an optimal level for sea lice development, buckets were placed in an environmental chamber with a temperature range of 10 ± 2°C during the culturing process; active copepodids were present 12 days post-hatch (Mustafa et al., 2000). A sea lice culture system consisting of a cylindrical container for egg string incubation and a one meter diameter tank for further development was employed by Walton (2008). Recently, a Norwegian research group published detailed information including photographs of the design of two types of sea lice culture systems: one small incubator system which was specifically designed for hatching individual pairs of egg strings, the other a large incubator mainly used for hatching and culturing larger numbers of *L. salmonis* copepodids (Hamre et al., 2009). In the examples provided above, the materials and equipment used for building the culture systems were inexpensive and the systems were relatively simplistic in design. The culture systems designed for the experiments described in Chapters 3 and 4 were customized based on the previously described designs.

The objectives of this non-experimental chapter were to: (1) design and establish a sea lice culture system in the laboratory by integrating knowledge of existing sea lice culture systems with several customized modifications; and (2) develop an enumeration

method for counting infective *L. salmonis* copepodids for the artificial parasitism of Atlantic salmon smolts required for Experiments 1 and 2 (Chapters 3 and 4, respectively). This chapter was not intended to investigate the effect of water temperature or other parameters on the hatching success of sea lice egg strings to copepodid stages.

2.3 Materials and Methods

2.3.1 Sea lice culture system design and establishment

The purpose of establishing a sea lice culture system was to obtain parasitic *L. salmonis* copepodids from field-collected *L. salmonis* egg strings for the subsequent artificial parasitism of Atlantic salmon (*Salmo salar*) smolts utilized in Experiments 1 and 2, involving cohabitation with cultured juvenile cunners (*Tautogolabrus adspersus*) (see Chapters 3 and 4, respectively). The sea lice culture system was established in the Aquaculture Facility of the Fisheries and Marine Institute of Memorial University of Newfoundland (MUN). This room was a secure dry lab with access to a stainless steel bench with running tap water and a drain, an aeraton system (air mainifold), a freshwater cooling supply (water chiller), a refrigerator (set at approximately 4°C) and overhead light source (fluorescent lights).

The sea lice culture system consisted of a closed seawater system (also referred to as sea lice culture units) comprised of: (1) small hatching incubators, (2) large grow-out

containers, (3) water bath tanks, (4) aeration manifold, and (5) water reservoir. The culture system was assembled as four independent sea lice culture units which were placed into two connected water bath tanks, with an aeration manifold consisting of eight individual air supply lines, allowing for control of air flow into individual sea lice culture units (Figure 2-1).

In terms of each sea lice culture unit, the small hatching incubators were constructed from 1.5 L plastic soda bottles with the bottom removed, and an air diffuser (flexible aquarium bubble wand) connected to an air line placed up through the bottle cap (Figure 2-2 (a), Table 2-1). Small holes (0.5-0.7 mm in diameter) were drilled round the middle area of the plastic soda bottle by using a 0.5 mm drill bit. The small hatching incubator was mounted upside-down within the large grow-out container by feeding plastic coated wires through the open edge of the pop bottle (Figure 2-3 (a); Table 2-1). The large grow-out containers were constructed from 18.9 L (5 gallon) plastic water jugs with the narrow top end removed. Handmade Banjo filters were constructed from a 2.5 cm ring cut from PVC pipe (2 inches in diameter) and sealed on both sides with an 80 micron nylon filter mesh connected with a 1/8 inch hose adaptor. The Banjo filters were installed inside the large grow-out containers on the bottom inside wall. A 1/8 inch hose was connected to the Banjo filter and clamped to the top edge of the large grow-out container. The Banjo filters were used for water level control and water exchange in the

large grow-out container (Figure 2-3 (b); Table 2-1).

The water bath tanks were constructed from two plastic tray-shaped containers, which were connected by a 1/4 inch PVC pipe. The water inlet and outlet were placed at each end of the water bath tank. There was a 50 gallon water bucket used as a water reservoir which was placed beside the water bath tanks. This reservoir was filled with tap water which was first cooled to 5-7ºC by an external water chiller and then pumped into the water bath tanks through the water inlet. Once the water bath tank was filled with tap water to the level of the outflow pipe, the additional tap water overflowed back into the water reservoir. The sea lice culture units were then partially submerged in the chilled water bath, which ensured a consistent water temperature was maintained between 11 ± 1ºC in the sea lice culture units (Figures 2-1 & 2-2 (a)). The lighting in the room was set for a photoperiod of 12hr light:12hr dark.

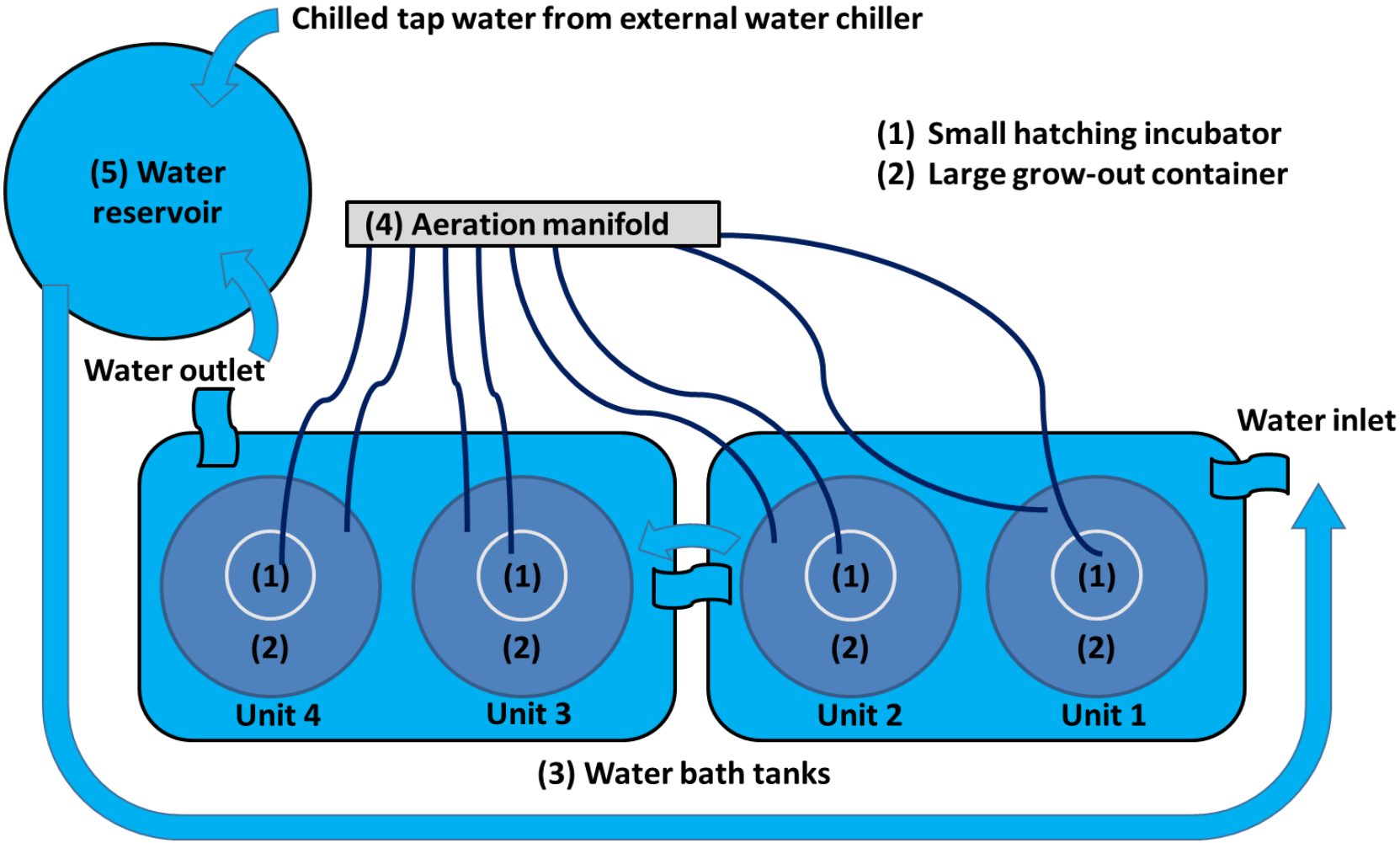

Figure 2-1**.** Schematic of the sea lice culture system.

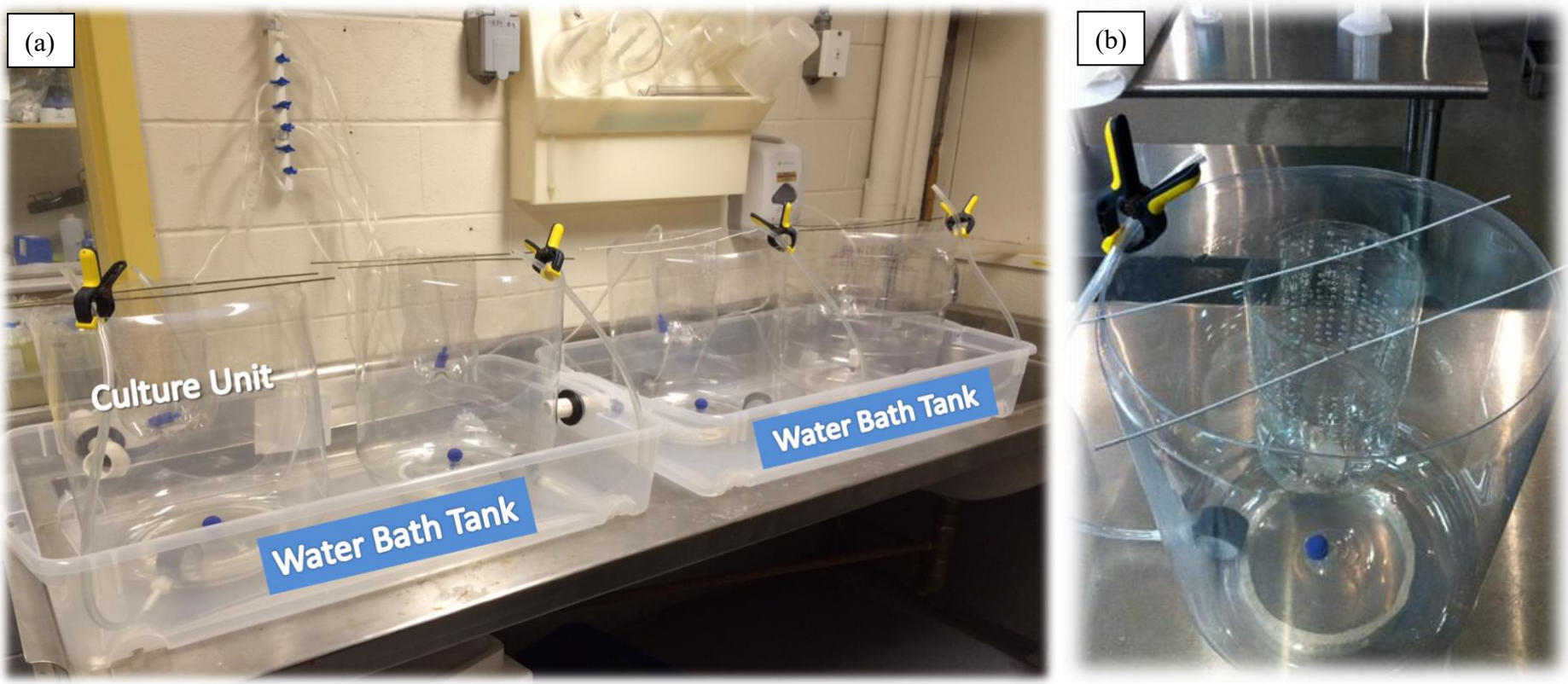

Figure 2-2. Pictures of the sea lice culture system: (a) All components of the sea lice culture system; (b) Individual sea lice culture unit.

Table 2-1. Key features of the sea lice culture units.

CULTURE UNIT	VOLUME	FEATURES
Small hatching incubators	1.5 L	• Area for egg string storage and incubation • 0.5-0.7mm holes drilled around center of incubator (see white box on Figure 2-3 (a)) to allow hatched nauplii to swim into large grow-out container • Nylon mesh (80 μm) covered hole at the bottom (to contain nauplii and empty shells during molting) (see white arrow on Figure 2-3 (a)) • Individual air stone placed inside; connected to manifold for individual control of air flow
Large grow-out containers	18 L	• Area for further development of hatched nauplii to copepodids • Tube connected Banjo filter (80 μm nylon mesh) for water level control (see white box on Figure 2-3 (b)) • Individual air stone centrally attached at the bottom; connected to manifold for individual control of air flow (see white arrow on Figure 2-3 (b))

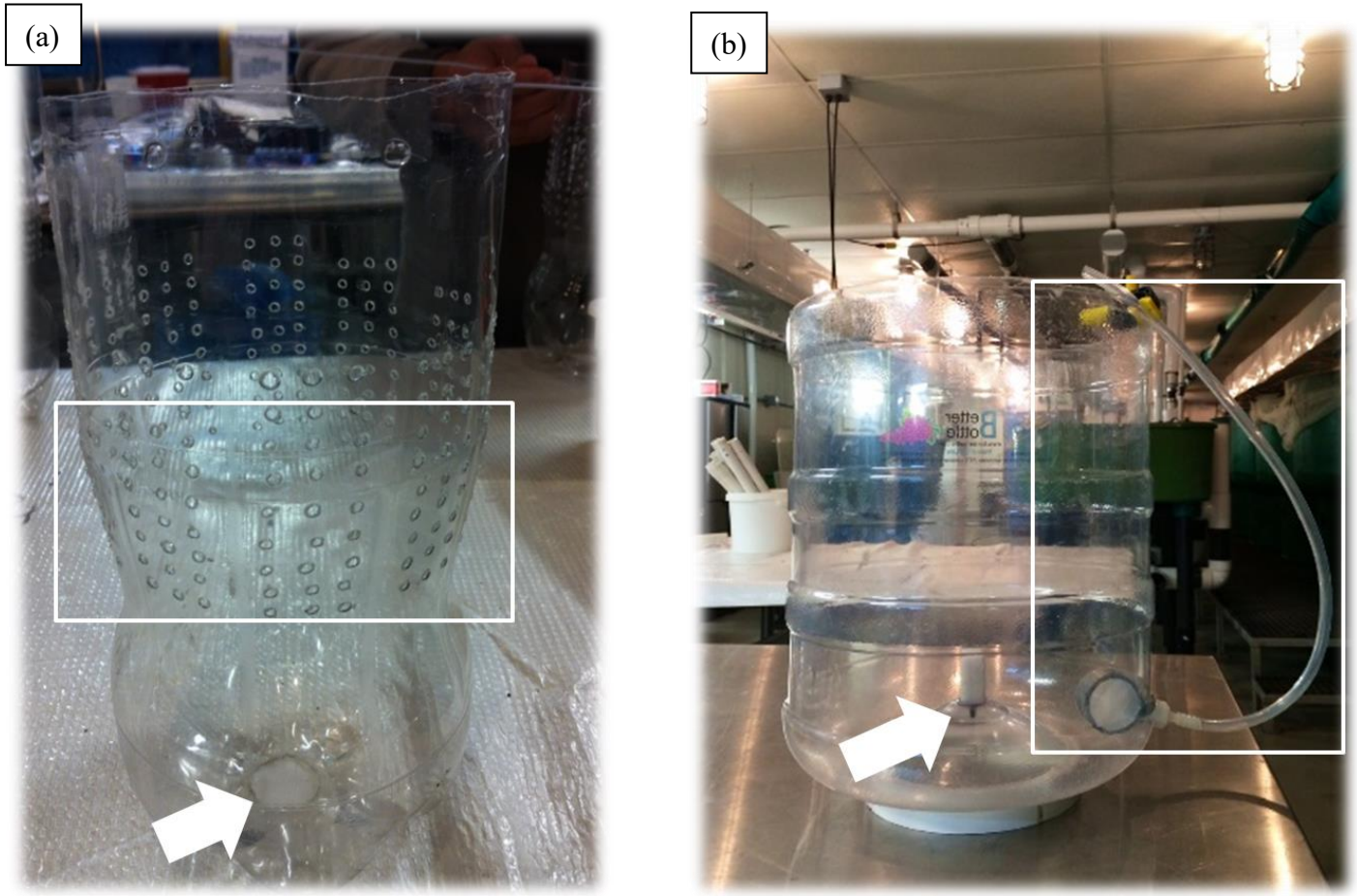

Figure 2-3. (a) Small hatching incubator (white arrow indicates hole covered with 80 µm nylon mesh) and (b) large grow-out container components of the individual sea lice culture units (white arrow indicates air stone centrally attached at the bottom).

2.3.2 Sea lice collections

Egg-bearing adult female (gravid females) *L. salmonis* were collected from two commercial Atlantic salmon grow-out sea cage sites which were located within the same Bay Management Area (BMA) in the Coast of Bays region, Newfoundland and Labrador (DFO, n.d.) on July 14th and Sept. 18th, 2016 during harvesting events. Fish had not recently been treated with chemotherapeutants and the average sea lice burden on fish was unknown at the time of collection. Sea lice were collected on board of a harvest boat from market-sized Atlantic salmon that were immobilized via percussive stunning. Individual gravid females were manually removed using fine-tipped forceps and placed into one of three 2 L Rubbermaid® plastic collection containers, which were filled with seawater ranging between 10-12°C which was obtained from the site at the time of the sea lice collection. All three containers were individually supplied with aeration (via a small air stone connected to a battery powered aquarium pump) and stored in a larger plastic cooler filled with ice packs, to ensure sea lice samples were kept cool during transport to the laboratory (approximately 10 hours). Upon arrival at the laboratory, the sea lice collection containers were placed in a 8-9°C water bath allowing gravid females to acclimate overnight, prior to the removal of egg strings the following morning.

2.3.3 Sea lice cultivation

Egg strings were manually removed from gravid females using forceps and scissors. They were then separated into two categories based on a subjective assessment of colour: dark brown and light yellow/beige; the dark color of the egg strings suggests that sea lice eggs are further along in development and more likely to hatch first, and the light ones are newly extruded fertilized egg strings (Boxaspen and Næss, 2000). Dark and light egg strings were separated by placement into different small hatching incubators within the large grow-out containers. The number of individual egg strings placed into each hatching incubator was not quantified. Each batch of egg strings was suspended in the small hatching incubators, containing 33 ppt seawater, by a gentle upflowing of air bubbles generated by the air stone placed in the bottom of the large grow-out container. Each large grow-out container was partially submerged in the water bath tank which contained running chilled tap water. The water temperature of the water bath tanks was maintained at approximately 7ºC during the cultivation procedure. The banjo filter was used to drain the sea lice culture units daily to approximately 30-35% of their volume, the volume was restored by adding seawater collected from Logy bay near the Ocean Sciences Centre (OSC). Upflowing air bubbles from the bottom of both small hatching incubators and large grow-out container was supplied via the aeration manifold to ensure egg strings were suspended in the water column.

The sea lice cultivation process occurred from Jul. 15th to Aug. 2nd, 2016 for Experiment 1 (see Chapter 3) and Sept. 18th to Oct. 2nd, 2016 for Experiment 2 (see Chapter 4). Due to the fact that the egg strings were collected from the field, there was no control over the stage of development of the eggs within the individual egg strings collected. As such, there was individual variation in the timing of egg string hatching and development in the laboratory within each sea lice culture unit and between units following each collection. A crude estimation of percentage of *L. salmonis* in each developmental stage within each operating culture unit was recorded during the cultivation process. The recorded developmental stages included (1) Egg strings (light & dark), (2) Nauplius I, (3) Nauplius II and (4) Copepodid.

2.3.4 Sea lice enumeration

The main purpose of this procedure was to quantify the approximate number of copepodids present in the individual sea lice culture units. A modification of the sea lice enumeration methods previously described (Walton, 2008; Hamre et al. 2009) was employed. This was accomplished by counting the total number of copepodids present in small subsamples removed from a larger volume of the sea lice culture unit contents. This enumeration was required in order to approximate the number of copepodids required for the artificial parasitism of Atlantic salmon for Experiments 1 and 2 (see Chapters 3 and 4,

respectively).

As the sea lice within the individual culture units developed to the parasitic copepodid stage, copepodids (0.7-0.8 mm in length and 0.3-0.4 mm in width) were visible without the aid of a microscope and could be counted with the aid of a dissecting microscope. Samples were collected from indivual sea lice culture units on the dates indicated in Figures 2-4 to 2-7. To remove individual subsamples of water from the sea lice culture unit containing the sea lice, the contents of the sea lice culture units (approximately 18 L) were stirred to ensure equal distribution of the copepodids within the culture unit, prior to removing subsamples (to ensure the subsample was representative of the total volume of the culture unit). Using a turkey baster, a 1 L (1000 mL) sample was removed from the center of the sea lice culture unit from the total 18L volume, and stored in a 2 L glass beaker. Two to four small subsamples (100 mL, 0.1 L) were removed from the 1 L sample and then the remaining 600-800 mL were returned to the sea lice culture unit. The number of subsamples was determined according to the sea lice density in each unit (i.e., two subsamples were removed from less dense unit). The total number of copepodids in each subsample (100 mL, 0.1 L) was counted by pouring smaller volumes (approximately 10-20 mL) into a plastic Petri dish. The total number of copepodids in each subsample of the 100 mL samples (0.1 L out of 18 L, 1:180) was averaged by repeating the steps previously described. Therefore, the total number of

copepodids in an individual sea lice culture unit was extrapolated by multiplying the mean total copepodid number in the 0.1 L sample by 180. The numbers derived from this process were subsequently utilized to determine the number of copepodids available for the artificial sea lice parasitism required for Experiments 1 and 2 (see Chapters 3 and 4, respectively).

2.4 Results

For Experiments 1 and 2, two sea lice culture units were used and the overall performance of the sea lice culture system was deemed successful according to a crude estimation of successful hatching of sea lice and their subsequent development to the copepodid stage. A crude estimation of the percentage of *L. salmonis* developmental stages in each sea lice culture unit for each experiment was recorded throughout the culturing process. As anticipated, the general trend observed during the sea lice culture periods for Experiments 1 and 2 exhibited that Unit 1, containing dark egg strings only, took a shorter amount of time (within 24 hrs) to develop to infective *L. salmonis* copepodids compared to Unit 2, which contained mostly light egg strings.

Egg string incubation for Experiment 1 commenced on Jul. 15th, 2016. Approximately 78% of dark egg strings in Unit 1 developed to copepodids by Jul 26th, 2016 at 10 ± 1ºC (Figure 2-4). Approximately 50% of light egg strings in Unit 2 hatched

to the Nauplius I stage 6 days post-incubation (Figure 2-5). Egg string incubation for Experiment 2 commenced on Sept. 18th, 2016. Approximately 50-60% of egg strings in Unit 1 hatched to Nauplius but did not develop further. This may have been temperature related, due to the condition of the sea lice egg strings at the time of year of egg collection, or due to individual sea lice variability, but this cannot be confirmed.

Subjective observations were used to make a crude estimation of the percentage of *L. salmonis* developmental stages present in each sea lice culture unit for both experiments. The number of sea lice egg strings collected and cultured in Experiment 1 was greater than that in Experiment 2. Thus, proportionally the amount of active infective copepodids available for the artificial sea lice parasitism for Experiment 1 was greater than that for Experiment 2.

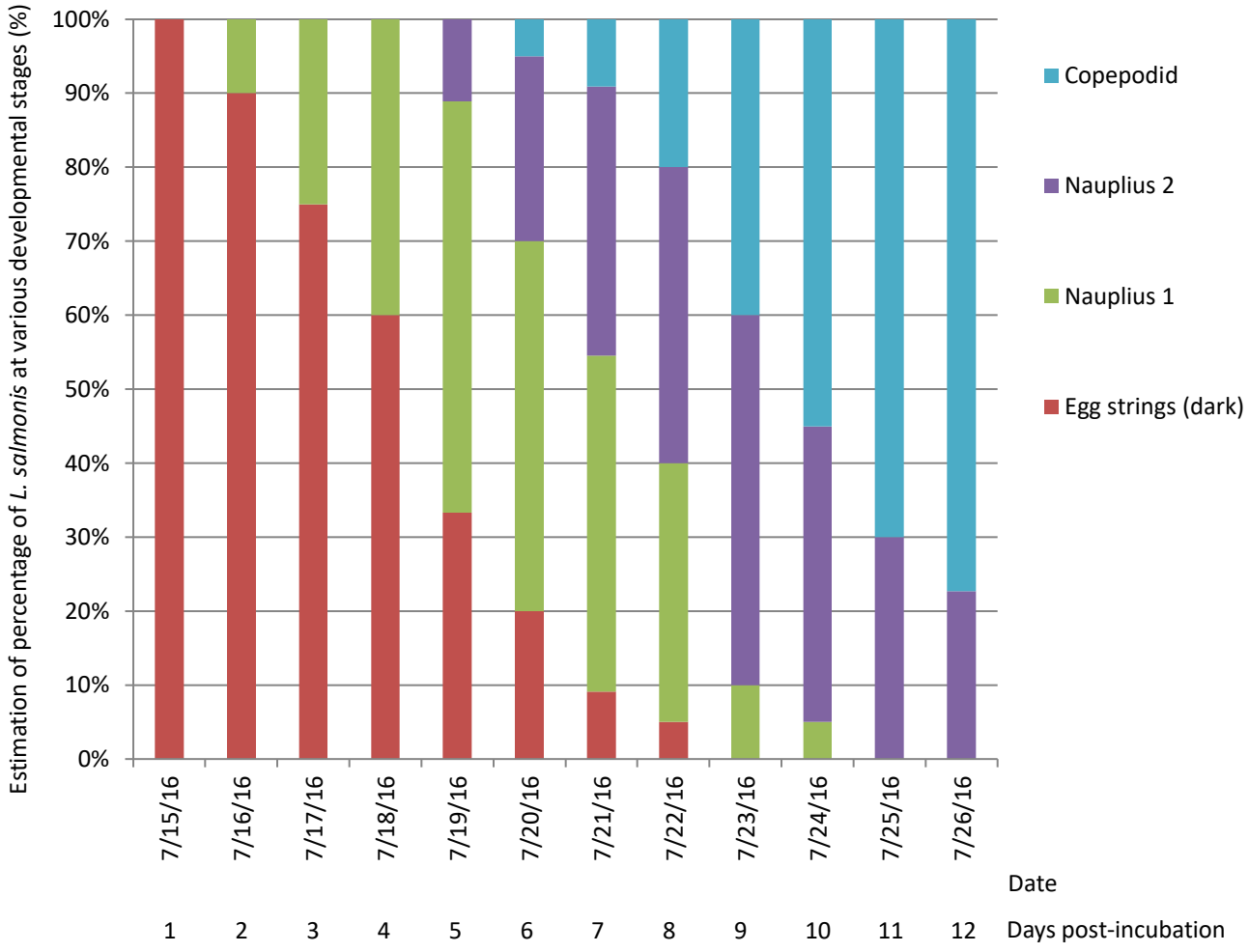

Figure 2-4. Crude estimation of percentage of *L. salmonis* developmental stages present in Unit 1 of the sea lice culture system from Jul. 15th to 26th, 2016 (Experiment 1) as determined by enumerating subsamples removed from Unit 1.

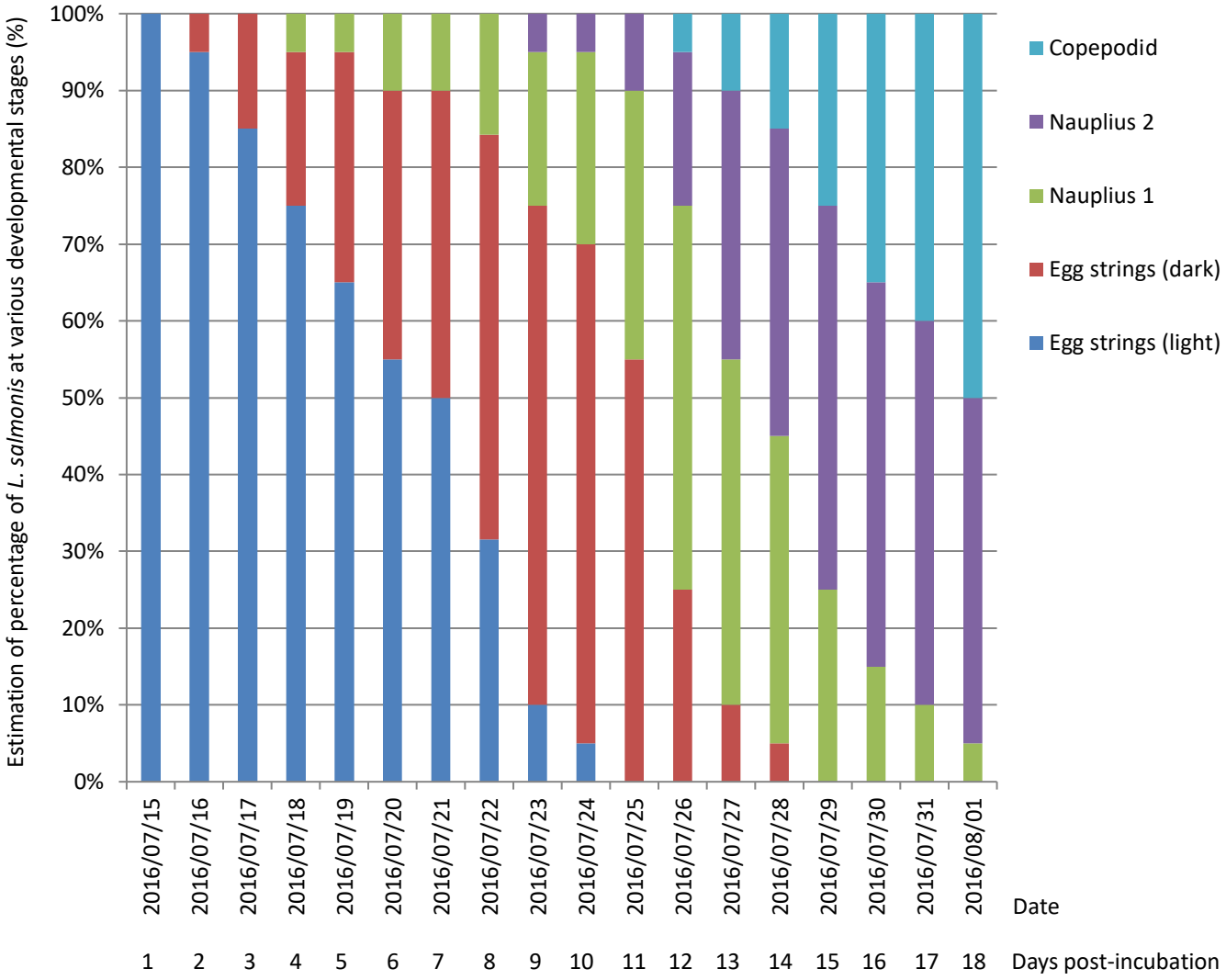

Figure 2-5. Crude estimation of percentage of *L. salmonis* developmental stages present in Unit 2 of the sea lice culture system from Jul. 15th to Aug. 1st, 2016 (Experiment 1) as determined by enumerating subsamples removed from Unit 2.

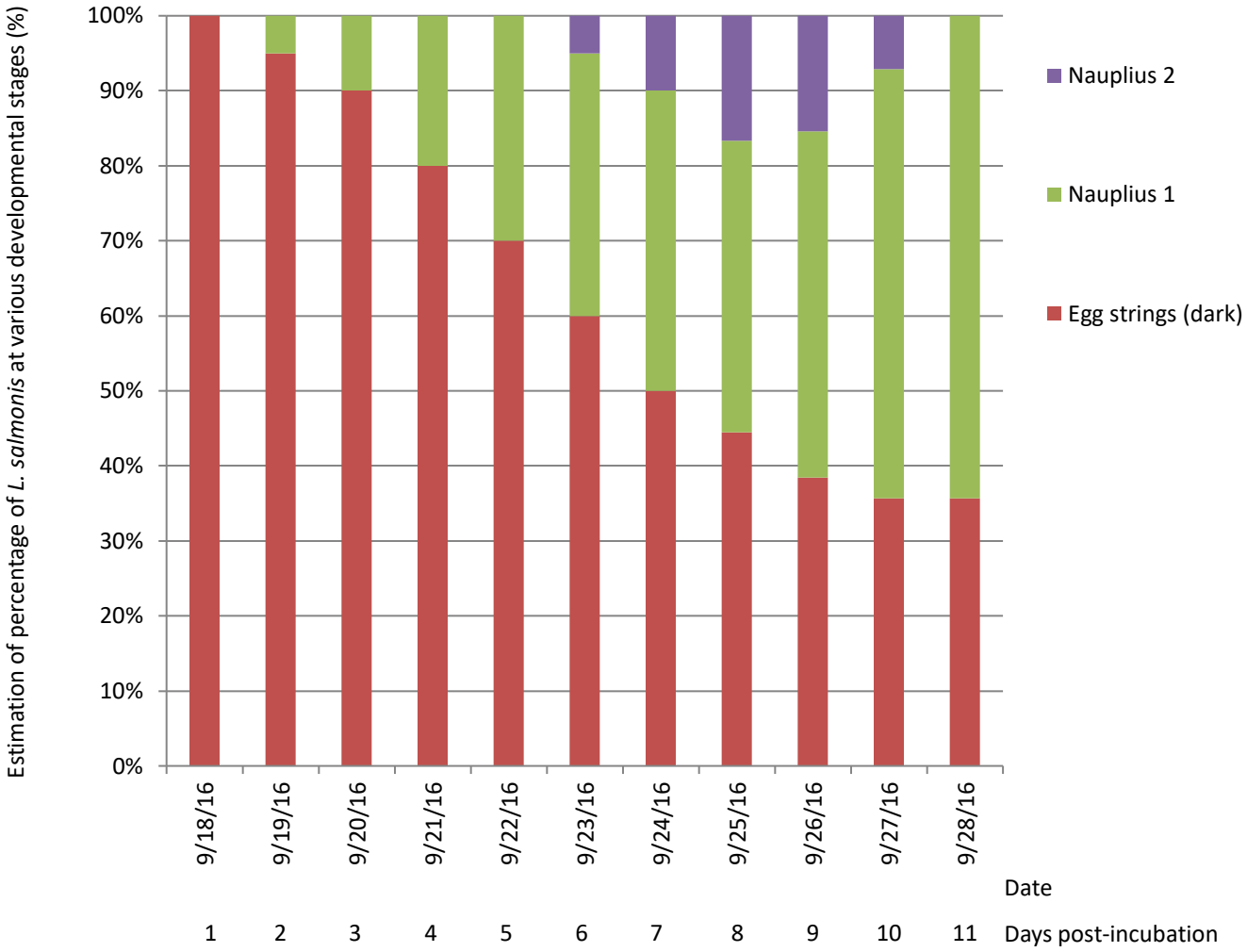

Figure 2-6. Crude estimation of percentage of *L. salmonis* developmental stages present in Unit 1 of the sea lice culture system from Sept. 18th to 28th, 2016 (Experiment 2) as determined by enumerating subsamples removed from Unit 1.

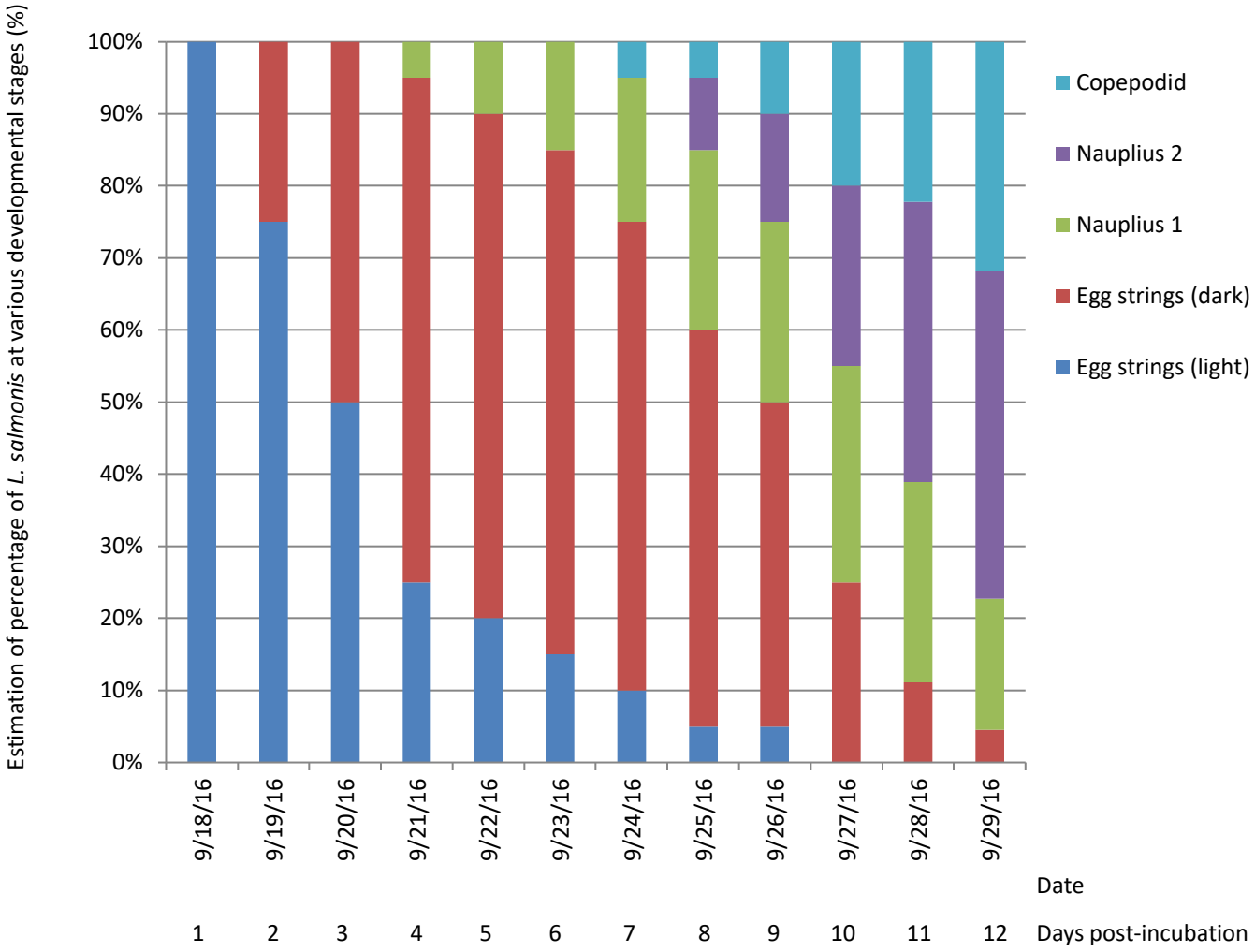

Figure 2-7. Crude estimation of percentage of *L. salmonis* developmental stages present in Unit 2 of the sea lice culture system from Sept. 18th to 29th, 2016 (Experiment 2) as determined by enumerating subsamples removed from Unit 2.

2.5 Discussion

2.5.1 Sea lice availability

There were a number of challenges encountered with respect to sea lice availability, resulting from the fact that the fertilized egg strings could only be obtained from field collections. Sea lice availability was dependent on season (late summer/fall is the preferred season), water temperature (as the sea lice life cycle is temperature dependent; higher sea lice numbers are present on fish at warmer water temperatures), and the harvest schedule of the participating company. The preferred location for the field collection of gravid female *L. salmonis*, to ensure access to the relatively high numbers, occured on a harvest boat where a large quantity of market size Atlantic salmon were removed from marine cage sites. Due to the gravid female size (8-11 mm in length), *L. salmonis* could be easily identified and picked from fish that were stunned percussively.

2.5.2 Evaluation of sea lice culture system

The sea lice culture system constructed and employed for the current work was required to supply infective *L. salmonis* copepodids for the artificial sea lice parasitism of Atlantic salmon described in Chapters 3 and 4. After exploring sea lice culture system design ideas from a number of research institutes (Grimnes & Jakobsen, 1996; Boxaspen & Næss, 2000; Walton, 2008; Mustafa et al., 2000; Hamre et al., 2009), the system

employed was constructed using inexpensive and readily available materials which were easily assembled and maintained. Due to the design of the laboratory, access to a flow-through seawater source was not possible and therefore the sea lice culture system had to be modified to a closed system. This was a significant challenge. The ideal design would include access to flow-through seawater. As such, the seawater had to be transported from the Ocean Sciences Centre (OSC), Logy Bay, Newfoundland, to the Marine Institute, as required. This necessitated a modification to the sea lice culture system to a closed seawater system comprised of four independent culture units in which seawater was stored and manually exchanged daily with a minimum daily exchange rate of 30% (of total volume) in each sea lice culture unit. To maintain the water temperature inside the culture unit, the culture unit was partially submerged in a water tank bath in which chilled tap water (approximately 7ºC) was continually circulating (see Figure 2-1). Additionally, seawater was chilled to approximately 6 ± 1ºC in a fridge before addition to the sea lice culture units. Minor short-term temperature fluctuations in sea lice culture units were likely to have occurred during daily exchanges of seawater within the sea lice culture units. This sea lice culture system ensured the stabilization of temperature and salinity for sea lice development during cultivation in the laboratory.

There was as failure to produce copepodids from the second sea lice collection (in Unit 1; Figure 2-6). It has been documented that the factors such as water temperature

and light could impact upon sea lice hatching and development to copepodid stages (Boxaspen & Næss, 2000). Due to the fact that the water temperature and photoperiod were consistent throughout all the sea lice hatching units for both collections, these parameters are unlikely to have contributed to the failure as discussed. Thus, parameter(s) that negatively affected egg string hatching are unknown.

2.5.3 Advantages and disadvantages of sea lice enumeration

A crucial step for artificial sea lice parasitism is to extrapolate the number of infective copepodids required to attain the desired sea lice challenge level for an experiment. Based on previously published research by Hamre et al. (2009), and the enumeration method described in this chapter, it was extrapolated that the desired challenge level for Experiment 1 was 75-90 infective *L. salmonis* copepodids per fish, which was anticipated to result in a final total sea lice number of approximately 25-30 pre-adult/adult *L. salmonis* per fish. The enumeration method that was developed allowed for the quick estimation (taking approximately 8-10 minutes per tank) of the total number of copepodids in each sea lice culture unit, and the subsequent attainment of the desired sea lice challenge level. A potential drawback to this method of enumeration is the fact that individual copepodids were not counted in the culture units (only a crude estimation was made) so the exact number of copepodids present was unknown. If the estimation of

copepodids in a culture unit by this method was inaccurate, this could have resulted in variability in sea lice challenge levels per fish between individual experimental tanks.

2.6 Conclusion

Overall, the sea lice culture system designed and employed for this research allowed for the successful development of *L. salmonis* egg strings through to infective copepodids under laboratory conditions. The sea lice culture system was functional, easy to operate, convenient to build, and suitable for research requiring artificial sea lice parasitism, which was subsequently employed in Experiments 1 and 2 in Chpaters 3 and 4, respectively The enumeration method proved to be suitable and was used for counting infective *L. salmonis* copepodids for the artificial parasitism of Atlantic salmon smolts for Experiments 1 and 2 in Chapters 3 and 4.

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CHAPTER 3. An evaluation of (i) the effect of stocking density on delousing efficacy of cultured juvenile cunner (*Tautogolabrus adspersus*) against *Lepeophtheirus salmonis* artificially infecting Atlantic salmon (*Salmo salar*) smolts and (ii) fish welfare (fin condition) of Atlantic salmon smolts during cohabitation

3.1 Abstract

While wild-caught cunner have been found capable of removing sea lice (*Lepeophtheirus salmonis*) from farmed Atlantic salmon (*Salmo salar*) under laboratory and field conditions, information regarding the ability of cultured cunner to remove sea lice when cohabitated with farmed Atlantic salmon is preliminary. Furthermore, there is a paucity in the published information regarding the stocking density required for favorable delousing efficacy and the potential influence on the fish welfare of farmed Atlantic salmon and cultured cunner in cohabitation. In order to investigate the delousing efficacy of cultured juvenile cunner against *Lepeophtheirus salmonis* artificially infecting Atlantic salmon smolts and fin condition (as a measure of fish welfare) of Atlantic salmon smolts during cohabitation, three stocking densities of cultured cunner (0, 4 and 10%) were co-habitated with Atlantic salmon smolts. Sea lice counts were conducted at T0 (pre-addition of cunner), 3 (T1), 5 (T2) and 7 (T3) days post-addition of cunner. Dorsal and caudal fins of Atlantic salmon smolts were assessed separately and assigned a score based on a 5-point fin erosion classification scale (i.e., 0, 1, 2, 3 or 4) at T0, T1 and T3. The mean dorsal and caudal fin erosion scores of Atlantic salmon smolts were calculated and compared across treatment groups over three sampling times (T0, T1 and T3). There was no significant effect of cultured juvenile cunner stocking density on the mean sea lice number per Atlantic salmon smolt per treatment group when held in cohabitation for

seven days (p=0.143). Interspecies interactions between cultured juvenile cunners and Atlantic salmon smolts were visually observed throughout the experiment, including some cunners occasionally approaching or swimming in the proximity of Atlantic salmon smolts, but delousing behaviour was not observed. Both statistical results and visual assessments suggested that the group of cultured juvenile cunners employed in this experiment did not exhibit delousing activity during cohabitation. Although not attributable to a treatment effect, the mean sea lice number per Atlantic salmon smolt in each of the three treatment groups (i.e., Control: 0% cunner; Density 1: 4% cunner and Density 2: 10% cunner) decreased significantly from T0 (pre-addition of cunner) to T3 (7 days post-addition of cunner) (p<0.001). There was no significant effect of cultured juvenile cunner stocking density on mean dorsal fin erosion score (p=0.463) and mean caudal fin erosion score (p=0.591) per Atlantic salmon smolt per treatment group when held in cohabitation for 7 days. This suggested that 4 and 10% cunner had no significant impact on dorsal and caudal fin condition of Atlantic salmon smolts during cohabitation under laboratory conditions.

3.2 Introduction

Cunner (*Tautogolabrus adspersus*) have been identified as a promising cleaner fish species for the Atlantic salmon aquaculture industry in Atlantic Canada. While the

delousing ability of wild-caught cunner against *Lepeophtheirus salmonis* and their stocking density when held in cohabitation with Atlantic salmon have been reported in several preliminary laboratory and field trials (DFO, 2014; Costa et al., 2016), no published information exists regarding an evaluation of their welfare when held in cohabitation with Atlantic salmon. Regarding cultured cunner, there is a paucity in information related to their delousing ability towards *L. salmonis*, the effect of stocking density on their delousing efficacy, and potential impacts on fish welfare when farmed Atlantic salmon smolts and cultured juvenile cunner are held in cohabitation.

The delousing efficacy and cost of using cleaner fish are closely associated with cleaner fish stocking density or ratio (Brooker et al., 2018; Powell et al., 2018). Various stocking densities or ratios have been investigated for their effect on the delousing efficacy of wild-caught cleaner fish used for sea lice control in Atlantic salmon aquaculture in Europe. In the 1990's, stocking densities of wild-caught wrasse employed in early commercial farm trials, investigating the use of various cleaner fish species to control different species of sea lice on Atlantic salmon, were relatively lower than current practices; ranging from as low as 0.4-0.6% (i.e., 1 wrasse: 150 salmon or 1 wrasse: 250 salmon) to 1-2% (i.e., 1 wrasse: 100 salmon or 1 wrasse: 50 salmon) (Deady et al., 1995; Tully et al., 1996). However, the objective of these studies was not to explicitly investigate the impact of various stocking densities on louse removal so the densities

varied. In laboratory trials with tank-based systems, the stocking density of wild-caught cleaner fish was higher than those deployed in the commercial farm trials previously described. For example, in order to investigate the delousing of *C. elongatus* on Atlantic salmon by two wrasse species (goldsinny and rockcook), Tully et al. (1996) employed a cleaner fish stocking density of 66%, stocking ten rockcook and ten goldsinny wrasse with 15 Atlantic salmon smolts, respectively, in two separate 1000 L tanks.

More recent laboratory and commercial field trials have been conducted to investigate the effect of stocking densities of both wild-caught and cultured cleaner fish (wrasse and lumpfish) on sea lice (*L. salmonis*) removal from Atlantic salmon in Norway and Scotland. For example, in a field trial carried out in sea cages (5.5 × 5.5 × 7 m) in Norwegian waters in order to investigate the delousing ability of cultured and wild-caught ballan wrasse, Skiftesvik et al. (2013) determined that, when stocked at a density of 5% wrasse (a ratio of 25 wrasse: 500 Atlantic salmon per experimental sea cage), both cultured ballan wrasse (*Labrus bergylta*) and a mixture of wild-caught ballan and corkwing wrasse (*Symphodus melops*) maintained average numbers of 'mobile' stages of *Lepeophtheirus salmonis* (i.e., pre-adult and adult stages) below one louse per Atlantic salmon (mean weight 429 ± 115 g), suggesting cultured ballan wrasse exhibited an equivalent delousing efficacy at a 5% stocking density compared to wild-caught ballan and corkwing wrasse stocked at the same density. In addition, stocking densities of a

mixture of wild-caught wrasse species tested in farm trials in Scotland ranged from 4-5% (1 wrasse: 21 Atlantic salmon to 1 wrasse: 27 Atlantic salmon) (Treasurer, 2013). In a laboratory trial in Scotland, Leclercq et al. (2014) investigated the delousing efficiency (*L. salmonis*) of three sizes of cultured ballan wrasse: small (114 ± 0 mm, 23.3 ± 0.4 g), medium (136 ± 1 mm, 43.4 ± 0.4 g) and large (164 ± 1 mm, 74.6 ± 0.5 g), each stocked at a density of 5% with 60 Atlantic salmon post-smolts (mean weight 137-150g) and compared to a negative control group (60 Atlantic salmon with 0% cultured ballan wrasse). They found that, when stocked at a density of 5%, all three sizes of cultured ballan wrasse significantly reduced "mobile" lice per salmon from 12-13 to below 0.5 during an 84-hour cohabitation period when compared to the negative control group.

Research has also been conducted on wild-caught and cultured lumpfish as a cleaner fish species where higher stocking densities have been employed. Imsland et al. (2014; 2015) investigated lumpfish (*Cyclopterus lumpus*) (derived from wild-caught broodstock) stocking densities of 10 and 15% (12 lumpfish: 120 Atlantic salmon and 18 lumpfish: 120 Atlantic salmon, respectively) in several field trials in Norwegian waters, demonstrating signs of lumpfish grazing (i.e., reduction in sea lice counts across 5 life stages) on *L. salmonis*. Currently, Norwegian Atlantic salmon farms stock various cleaner fish species at ratios of up to 12 to 20% (Treasurer, 2018), the UK up to 5-10% (Treasurer et al., 2018), and Ireland up to 4-6% (Bolton-Warberg, 2018) for sea lice (*L. salmonis*)

control.

With respect to the use of cunner (wild-caught or cultured) as a cleaner fish species on Atlantic salmon sea cage sites in Canada, the stocking density required for optimal delousing and fish welfare of both species of fish is currently unknown. While this particular aspect of wild-caught and cultured cunner has been under scientific investigation, limited information has been published to date. An early study conducted by MacKinnon (1995) investigated the delousing potential of wild-caught cunner (size not specifically indicated; most were larger than 10cm) on sea lice (*Caligus elongatus*) removal from Atlantic salmon smolts under laboratory conditions and in subsequent sea cage trials. Under laboratory conditions, one wild-caught cunner was held in a 136 L glass aquaria with one small cultured Atlantic salmon smolt (18-22 cm in length) (i.e., 100% cunner stocking density) which was artificially infected (50 adult stage sea lice collected from naturally infested salmon were placed into a separate 5 L plastic container with the Atlantic salmon smolt for a fifteen minute exposure period to allow for sea lice attachment) with *Caligus elongatus* and compared to a control group (two Atlantic salmon smolts without cunner; 0% cunner). A significant reduction in sea lice was detected after 24 hours of cohabitation (from 2.4 to 0.4 sea lice per fish, $p<0.05$). However, the results also demonstrated that not all wild-caught cunner removed sea lice at a density of 100% and delousing behaviour was not consistently exhibited by all

experimental cunner populations. Subsequently, the researcher conducted a 12-week field trial on a commercial sea cage site to evaluate a stocking density of 1.5% wild-caught cunner (i.e., 30 cunner: 2000 Atlantic salmon smolts) for the removal of *C. elongatus* from Atlantic salmon smolts. The results showed that there was no significant difference in sea lice numbers on Atlantic salmon between sea cages with or without cunner present. This suggested that a stocking density of 1.5% cunner was not effective for removing *C. elongatus* from Atlantic salmon smolts (MacKinnon, 1995).

In 2014, a Canadian Technical Report (DFO, 2014) described laboratory research and a subsequent field trial conducted by Kelly Cove Salmon Ltd. in New Brunswick (NB), Canada. The behaviour of wild-caught cunner and Atlantic salmon when held in cohabitation (laboratory trial) was investigated to determine the interest of the wild-caught cunner in consuming pelleted feed (laboratory trial), and the delousing ability of wild-caught cunner (field trial). The two separate six-month field trials (Trial 1 from September, 2011 - March, 2012; Trial 2 from April, 2012 - October, 2012), evaluated four stocking densities (3%, 6%, 9% and 12% cunner) of wild-caught cunner (lengths and weights not reported) to determine an optimal density for the removal of *L. salmonis* from Atlantic salmon (approximately 2 kg in weight). The researchers suggested that stocking densities of 9 and 12% wild-caught cunner might not be economically viable or environmentally sustainable due to the infancy of the cunner fishery in Canada at that

time.

Another Canadian laboratory trial studied wild-caught cunner (average length of 14.7 cm, average weight not indicated) delousing behaviour at a stocking density of 10% cunner (5 cunners: 50 Atlantic salmon smolts) when cohabitated in two 1364 L tanks; one tank containing Atlantic salmon and no cunner was used as the control tank. The sea lice challenge involved the collection of adult *L. salmonis* from Atlantic salmon sea cage sites of Newfoundland and Labrador, Canada. Sea lice were added to the three tanks containing the Atlantic salmon to initiate the artificial parasitism; tanks were lowered to 50% capacity and 300 adult sea lice were added to each of the three tanks for a 1-hour exposure period. During the course of experiment, cunner behaviour was documented, including cunner chasing Atlantic salmon smolts artificially infected with *L. salmonis* and multiple louse picking attempts, which were all considered positive sea lice cleaning behaviours. However, no significant reduction of *L. salmonis* numbers on Atlantic salmon was detected in the two experimental tanks when compared to the control tank (0% cunners) (p=0.275) within a 78-hour cohabitation period, based on an assessment of the change in sea lice counts or cunner behaviour over time in each tank (Costa et al., 2016).

Stocking density of cleaner fish is an important consideration with respect to its potential to impact upon the welfare of the cleaner fish and cultured species when held in cohabitation. In a shared aquatic environment, whether or not the cohabitation of Atlantic

salmon and cleaner fish species has an impact on the fish welfare of either species remains uncertain. Driven by the development of Integrated Pest Management (IPM) for sea lice control, an increasing amount of attention has been paid to fish welfare during the application of cleaner fish treatments in Atlantic salmon aquaculture (Treasurer & Feledi, 2014; Treasurer, 2018). While operational and laboratory-based welfare indicators have been proposed for some cleaner fish species such as lumpfish (e.g., active fin damage, sores, eye damage, opercula damage, suction disc deformities, etc.) (Noble et al., 2019), published information is lacking for cultured cunner. The assessment of several fin erosion indices have been applied to salmonids, such as Atlantic salmon (MacLean et al., 2000) and rainbow trout (Hoyle et al., 2007) as a measure of fish welfare. Fin erosion has been assessed by a range of methods using various indices. Factors known to cause fin erosion when holding cleaner fish with Atlantic salmon during deployment in sea cages include potential aggressive inter-species interactions (Leclercq et al., 2014) and sub-optimal stocking density (Treasurer, 2018). While various welfare indices have been proposed for cleaner fish, there are currently no universally adopted indices. However, fin condition indices have been suggested for cleaner fish deployed for sea lice control on Atlantic salmon farms (Treasurer & Feledi, 2014). Preliminary research by Treasurer & Feledi (2014) examining the physical condition and welfare of five species of wrasse, used a 5-point classification scale to assess the erosion and splitting of dorsal, pectoral,

anal and caudal fins, as measured by a fin erosion index (FEI) and fin splitting index (FSI).

The objectives of this chapter were: (1) to investigate the effect of stocking density of cultured juvenile cunner (*Tautogolabrus adspersus*) on their delousing efficacy when cohabitated with Atlantic salmon (*Salmo salar*) smolts artificially infected with sea lice (*Lepeophtheirus salmonis*) and, (2) to investigate the effect of stocking density of cultured juvenile cunner on fin condition (e.g., fin erosion score of dorsal and caudal fins) of Atlantic salmon smolts in cohabitation.

3.3 Materials and Methods

3.3.1 Laboratory rearing conditions

This experiment was conducted in the Aquaculture Facility of the Fisheries and Marine Institute (MI) of Memorial University (MUN), in a saltwater recirculating aquaculture system (RAS). The system used contained nine 1.5 cubic meter tanks (1500 L), each containing 700 L of saltwater (31-33 ppt), initially maintained at 11 ± 1ºC with individual level control through the use of a typical "stand pipe" approach. Each stand pipe was connected to a 4" effluent line that never exceeded 50% capacity. The effluent lines flow to vortex separators where approximately 90% of solids are removed followed by additional water polishing in Hydrotech Drum Filters containing 19 micron filter screens. This system removes all particles greater than 19 microns and ensures no cross

tank contamination. The saltwater was collected from Logy Bay which is adjacent to the Dr. Joe Brown Aquatic Research Building (JBARB) of the Ocean Sciences Center (OSC) at MUN, NL. Saltwater was delivered by truck to MI on a weekly basis, or more frequently as required. The photoperiod was 12h light: 12h dark. System water temperature, dissolved oxygen (DO), salinity, pH, and unionized ammonia and nitrite levels were measured daily. All water quality parameters remained in the normal range for Atlantic salmon smolts throughout the course of the experiment. Each tank was covered with a black mesh net to prevent fish from jumping out. There was one additional tank for temporarily housing the cultured juvenile cunners, prior to their addition to experimental tanks which followed the artificial sea lice parasitism as described below in section 3.3.3. To mimic and optimize the rearing conditions for cultured juvenile cunners, four artificial "hides" were deployed in the tank to simulate a shelter of kelp or a coral reef in the ocean. Each "hide" was made of a plastic ring, which was a cross-cutting of a 3-inch PVC pipe, with long strips of black plastic tarp tied around the plastic ring (Appendix C). These hides were not added to the experimental tanks for the subsequent sea lice challenge.

3.3.2 Experimental fish

The animals were handled and cared for in accordance with the Canadian Council

on Animal Care's Guidelines on the Care and Use of Fish in Research, Teaching and Testing (Canadian Council on Animal Care, 2005). The study was approved by the Animal Care Committee of Memorial University (ACP 15-02-JW).

Atlantic salmon smolts (256.4 ± 5.3 g, 28.3 ± 0.2 cm, Saint John River strain), were obtained from a commercial Atlantic salmon hatchery in Newfoundland and Labrador. They were held in the system for approximately 6 months prior to the start of the experiment to allow for the establishment of the biofilter. Smolts were equally allocated amongst nine experimental tanks (n=50 per tank). The experimental tanks were randomly assigned to one of three treatment groups (three tanks per treatment group). Prior to the start of the experiment, fish were hand-fed to satiation twice daily with Corey® Marine Aquafeed (4 mm) and the daily feed consumption per tank was recorded (although feed consumption data has not been presented, there was no indication of differences in feed consumption between tanks prior to the start of the experiment). Feeding was stopped when cunner were added to experimental tanks containing Atlantic salmon. Cultured juvenile cunners were supplied by JBARB, and temporarily housed under the same rearing conditions, as described above, in a separate tank within the same system containing the nine experimental tanks. The cultured juvenile cunners (age 1+ years) used in this experiment were the first generation progeny (F1 stock) of wild-caught broodstock reared in a land-based flow-through saltwater tank system at JBARB. Thie

cultured juvenile cunners were hand-fed to satiation every two days, using a mixture of two types of marine species diets: Skretting® North America Gemma Diamond (pellet size: 1.8 mm, Lot # 7220772) and Europa® (pellet size: 2.0 mm, Lot # 8601A).

3.3.3 Artificial sea lice parasitism

The water inflow to each 1500 L tank was turned off and the depth of the water in each tank lowered to approximately 15 cm. An air diffuser (rectangular air stone, 25 × 25 ×100 mm) was placed in each tank and used to deliver oxygen in order to maintain dissolved oxygen levels above 8 mg/L during the procedure. Saltwater containing a designated number of infective copepodids (see paragraph below and refer to section 2.3.4 in Chapter 2 regarding details of sea lice enumeration) were removed from the sea lice culture system and added to each experimental tank. The same artificial sea lice challenge procedure was followed for each experimental tank, until all nine experimental tanks were artificially infected. The monitoring of dissolved oxygen (DO) for each tank was initiated following the addition of copepodids, and the DO values were measured every 15-20 minutes with an oxygen meter (Handy Polaris, OxyGuard®). To maintain DO levels above 8 mg/L (approximately 80% saturation) during the 3-hour artificial sea lice parasitism, supplemental oxygen was added to each experimental tank and levels were adjusted accordingly based on DO levels measured every 15-20 minutes. After Atlantic

salmon smolts were exposed to the copepodids in the shallow and static saltwater environment for three hours, the water inflow was restored to refill the tank back to its original volume (approximately 700L). All nine experimental tanks were restored to their original tank volumes and the DO levels were monitored for an additional 30-40 minutes to ensure they remained at 8-9 mg/L.

In order to prevent a potential outcome of a higher than desired sea lice burden (e.g., over 30 mobile sea lice per fish as explained in Chapter 2) for the experimental fish population, a preliminary sea lice challenge was conducted in a manner such that all experimental tanks of Atlantic salmon smolts received a lower challenge level of approximately 1250-1300 *L. salmonis* copepodids per tank (as it was anticipated that this challenge level would result in approximately 10 mobile sea lice per fish) on July 27th, 2016, which was one third of the desiginated level (3750 *L. salmonis* copepodids per tank resulting in approximately 25-30 mobile sea lice per fish, see Chapter 2). In order to determine successful attachment of infectious *L. salmonis* copepodids, a small sample (n=3-5 Atlantic salmon smolts) from each tank were non-randomly selected, anesthetized using 4 mg/L TMS, and examined 6 days post-artificial parasitism (on August 2nd). Due to the fact that the attached copepodid numbers were lower than anticipated, a second sea lice challenge was conducted on the same day with approximately 3750 *L. salmonis* copepodids per tank, which was to ensure a sufficient number of infective *L. salmonis*

copepodids.

3.3.4 Experimental design and sampling schedule

Low (4%) and high (10%) cultured juvenile cunner stocking densities were chosen based on previously published studies and the proportion of Atlantic salmon in each tank (rounded to the nearest whole number for cunners). A random draw was used to ensure each tank had an equal probability of being assigned to each treatment group. All nine tanks were randomly assigned to three treatment groups: Control (0% cunner), Density 1 (4% cunner; 2 cunner: 50 salmon) and Density 2 (10% cunner; 5 cunner: 50 salmon). Each treatment group consisted of triplicate tanks.

Approximately 4 weeks after artificial sea lice parasitism, the attached *L. salmonis* copepodids further developed to the pre-adult and adult stages on Atlantic salmon smolts. Experiment 1 commenced on August 25th, 2016 (23-29 days post-incubation), the day prior to the addition of cunners (T0). In each tank, twenty five of the fifty Atlantic salmon smolts were evaluated using systematic random sampling. Under anaesthesia (4 mg/L TMS; MS-222), body weight (g) and fork length (cm) were measured; sea lice counts and fin erosion score (e.g., dorsal and caudal fins) were conducted by one individual through visualexamination of each sampled fish under bright light. The next day, the designated number of cultured juvenile cunner were added to

experimental tanks of the treatment groups (Density 1 and Density 2). Subsequently, three additional samplings of 25 fish/sample were conducted at 3 (T1), 5 (T2) and 7 days (T3) post-addition of cultured juvenile cunner. A 7-day experimental period was chosen due to logistical and financial constraints. Sea lice counts were performed during each sampling event (T0-T3) (Figure 3-1). Sea lice counts, which were initially categorized into three development stages (i.e., pre-adult, adult males and females), were combined and presented as the total sea lice number per fish due to the logistical constraints and time required for individual categorization of sea lice at each developmental stage during each sampling event.

For the current study, the 6-point classification scale (i.e., 0-5) for fin erosion developed by Hoyle et al. (2007), and the scales used by Person-Le Ruyet et al. (2007) and Treasurer and Feledi (2014), were modified to a 5-point fin erosion scale (i.e., 0-4) to assess dorsal and caudal fins separately, as described previously,, where, 0=no erosion, 1=slightly eroded, 2=moderately eroded, 3=half fin eroded, and 4=severely eroded (Appendix D). Additionally, a fin erosion photographic identification key (adopted from Hoyle et al., 2007 and Person-Le Ruyet et al., 2007) was used to provide assistance during the visual assessment of Atlantic salmon fin condition.. The dorsal and caudal fins were assessed separately at T0 (the day prior to the addition of cunner; this assessment was used to elucidate the initial fin condition before deployment of cultured juvenile

cunner), T1 (3 days post-addition of cunner) and T3 (7 days post-addition of cunner). There was no a 5 day scoring of the dorsal and caudal fins in an effort to minimize the handling of Atlantic salmon smolts. The mean fin erosion score was firstly calculated within each experimental tank and then further averaged within the same treatment group.

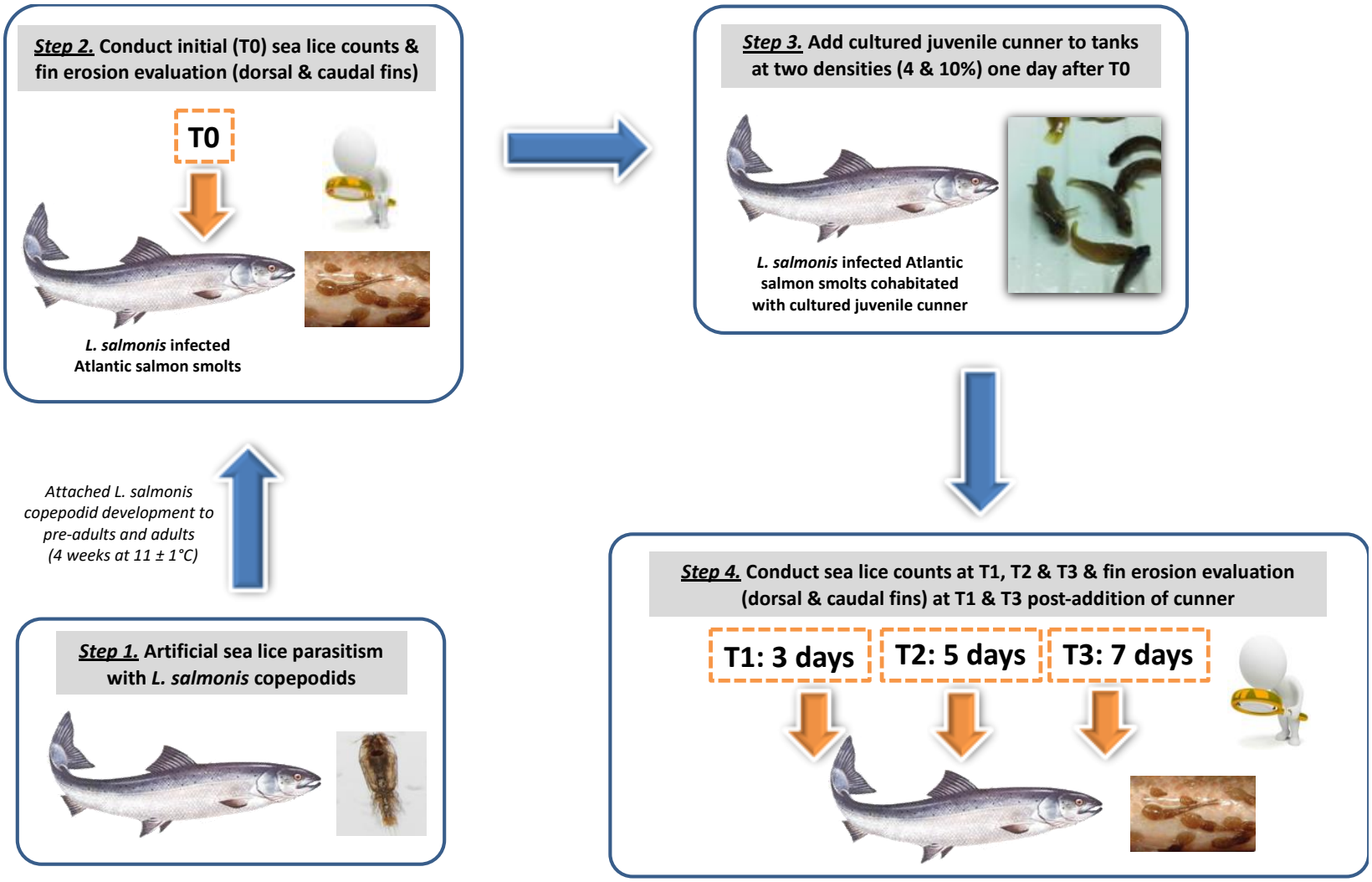

Figure 3-1. Experimental design and procedures.

3.3.5 Statistical analysis

Normality of distribution, the homogeneity of variance, and residuals plots of the raw data was assessed to determine the overall fit of the regression model using Minitab® 17 (Minitab 17 Statistical Software, 2010). A linear regression model was conducted using STATA/SE™ 15 (special edition) statistical software (StataCorp, 2017) to evaluate the effect of treatment, time (days 0, 3, 5 and 7 days post-cunner introduction), and tank on the mean sea lice number on Atlantic salmon smolt by tank sampled at 0, 3, 5 and 7 days post-addition of cultured juvenile cunner. The mean sea lice number per Atlantic salmon smolt per treatment group was assessed by one-way analysis of variance (ANOVA) and the significance among treatment groups within the same time period and the significance among treatment groups across sample periods was compared using a Tukey's multiple comparison test (Minitab 17). Based on the results on the normality test, only one standard error of the mean is displayed in each figure. A linear regression model was conducted using STATA/SE 15 to test for the effect of treatment, time of sampling and tank on the mean fin erosion score (e.g., dorsal and caudal fins) per tank at 0, 3 and 7 days post-addition of cultured juvenile cunner. The anlaysis was conducted by tank at 0, 3 and 7 days post-addition of cultured juvenile cunner. The mean fin erosion score (e.g., dorsal and caudal fins) in the Atlantic salmon smolt was assessed using a one-way analysis of variance (ANOVA) to compare significance between the treatment groups within the same time period (Minitab 17). Tukey's multiple comparison test was used to assess the significance between treatment groups across the sample periods (Minitab 17). Statistical differences were considered significant at $p<0.05$. To assist in the analysis, statistical significance among treatment groups within the same time period were denoted by lower case letters and those between treatment groups across sample periods were denoted by upper case letters.

3.4 Results

Based on the results of the regression anaylysis, there was no significant effect of cultured juvenile cunner stocking density on the mean sea lice number (pre-adult and adult stages combined) per Atlantic salmon smolt when held in cohabitation for seven days (p=0.143). Although subsequently deemed not attributable to a treatment effect, the mean sea lice number per Atlantic salmon smolt in each of the three treatment groups (Control:0% cunner; Density 1:4% cunner and Density 2:10% cunner) decreased significantly from T0 (pre-addition of cunner) to T3 (7 days post-addition of cunner) (p=0.000).

Atlantic salmon smolts in the three treatment groups did not have a similar mean sea lice number at T0 (pre-addition of cunner), as determined by a Tukey's pairwise comparison between treatment groups; the Control group (0% cunner), had a significantly lower initial mean sea lice number (18.3 ± 0.89) compared to Density 2 (10% cunner; 21.8 ± 1.20), but statistically similar to Density 1 (4% cunner) (20.9 ± 0.74), while Density 2 and Density 1 were not significantly different from one another (Figure 3-2).

The greatest reduction in the mean sea lice number per Atlantic salmon smolt per treatment group occurred during the first time interval from T0 (pre-addition of cunner) to T1 (3 days post-addition of cultured juvenile cunner), during which time the Control group decreased to 12.4 ± 0.71, Density 1 to 12.9 ± 0.66, and Density 2 to 12.7 ± 0.72.

Overall, the results of this experiment suggest that cultured juvenile cunner did not actively remove sea lice from Atlantic salmon smolts when held in cohabitation for 7 days while stocked at densities of 4 and 10%. However, a significant tank effect was detected (p<0.001) among the three treatment groups over the four sampling periods. All nine tanks demonstrated a decreasing trend in the mean sea lice number per Atlantic salmon smolt per tank from T0 to T3 (Figure 3-1). The mean sea lice number per Atlantic salmon smolt per tank was highly variable at each sampling point. Although a statistical

examination was not conducted on a tank level, when each tank was assessed individually (i.e., the mean sea lice number per Atlantic salmon smolt per tank) as an experimental unit, Tank 11 (Density 2, 10% cunner) had a higher mean sea lice number per Atlantic salmon smolt (29.0 ± 2.16) compared to the other experimental tanks at T0 (Figure 3-3). The reason for this is unknown, however, it might be due to natural variability inherent in live organisms. It should be noted that there was no sea lice count data for Tanks 13 and 15 at T3 due to human error associated with fish sorting into experimental tanks during the T3 sampling event.

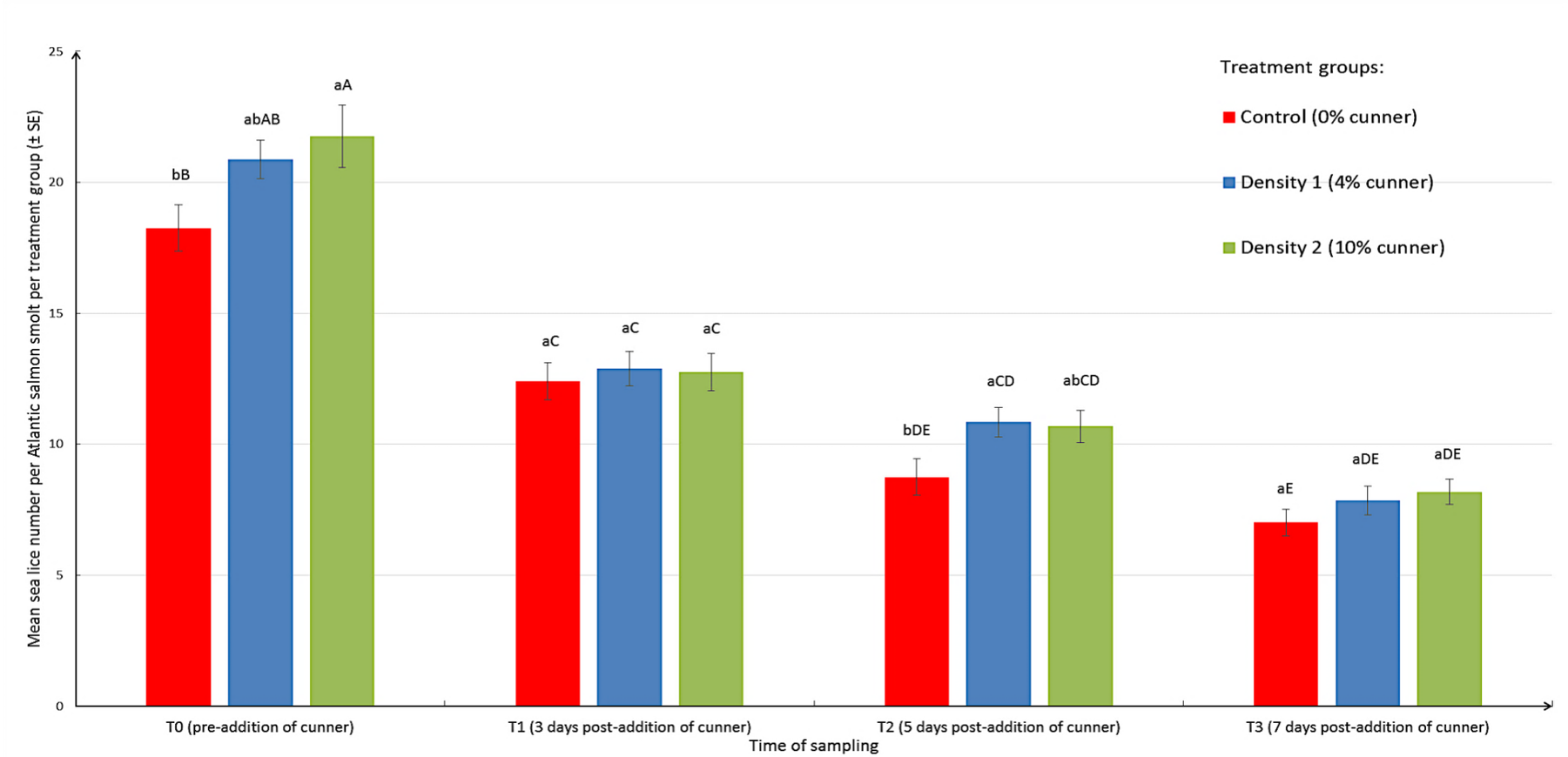

Figure 3-2. Mean sea lice (*Lepeophtheirus salmonis*) number on Atlantic salmon (*Salmo salar*) smolts sampled at 0, 3, 5 and 7 days post-addition of cultured juvenile cunner (*Tautogolabrus adspersus*) stocked at densities of 0, 4 and 10%. Values represent mean sea lice number per Atlantic salmon smolts per treatment group. Error bars represent plus and minus one standard error from the calculated mean. Lower case letters denote significance among treatment groups within the same time period. Upper case letters denote significance among treatment groups across sample periods.

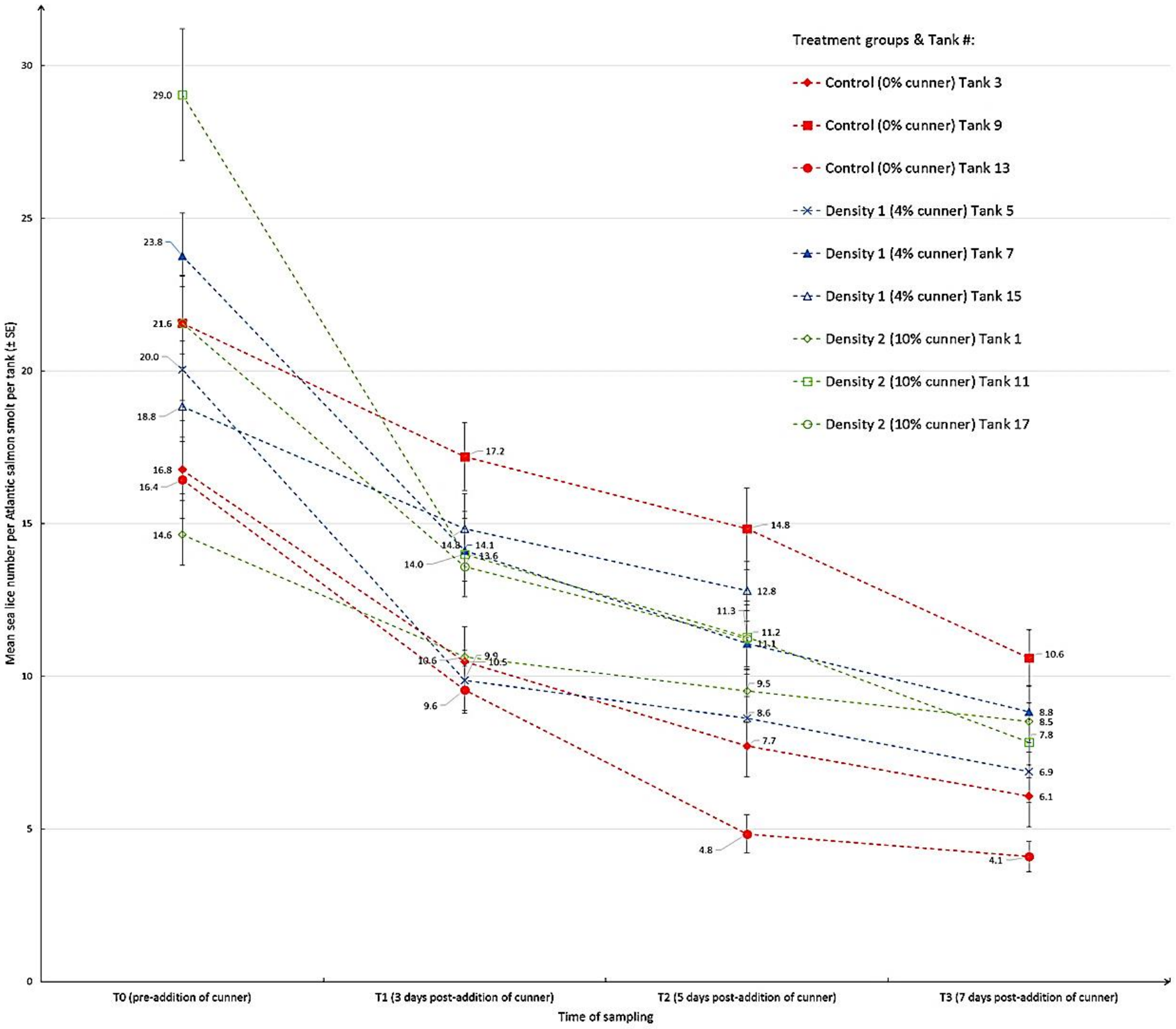

Figure 3-3. Tank level mean sea lice (*Lepeophtheirus salmonis*) number on Atlantic salmon (*Salmo salar*) smolts sampled at 0, 3, 5 and 7 days post-addition of cultured juvenile cunner (*Tautogolabrus adspersus*) stocked at densities of 0, 4 and 10% (treatment groups). Values represent mean sea lice number per Atlantic salmon smolt per experimental tank of treatment group. Error bars represent plus and minus one standard error from the calculated mean. There was no sea lice count data for Tanks 13 and 15 at T3 due to human error associated with fish sorting into experimental tanks during the T3 sampling event.

Based on the results of the regression anaylysis, there was no significant effect of cultured juvenile cunner stocking density on mean dorsal fin erosion score per Atlantic salmon smolt per treatment group when held in cohabitation for 7 days (p=0.463). This suggested that the dorsal fin condition of Atlantic salmon smolts was not affected by the addition of cultured juvenile cunner at stocking densities of 4 and 10% over a 7-day period of cohabitation. Additionally, neither time of sampling (p=0.463) nor experimental tank (p=0.185) had a significant effect on mean dorsal fin erosion score per Atlantic salmon smolt per treatment group (Figure 3-4).

There was no significant effect of cultured juvenile cunner stocking density on mean caudal fin erosion score per Atlantic salmon smolt per treatment group when held in cohabitation for 7 days (p=0.591). This suggested that the caudal fin condition of Atlantic salmon smolts was not affected by the addition of cultured juvenile cunner at stocking densities of 4 and 10% over a 7-day period of cohabitation. Additionally, neither time of sampling (p=0.390) nor experimental tank (p=0.188) had a significant effect on mean dorsal fin erosion score per Atlantic salmon smolt per treatment group (Figure 3-5).

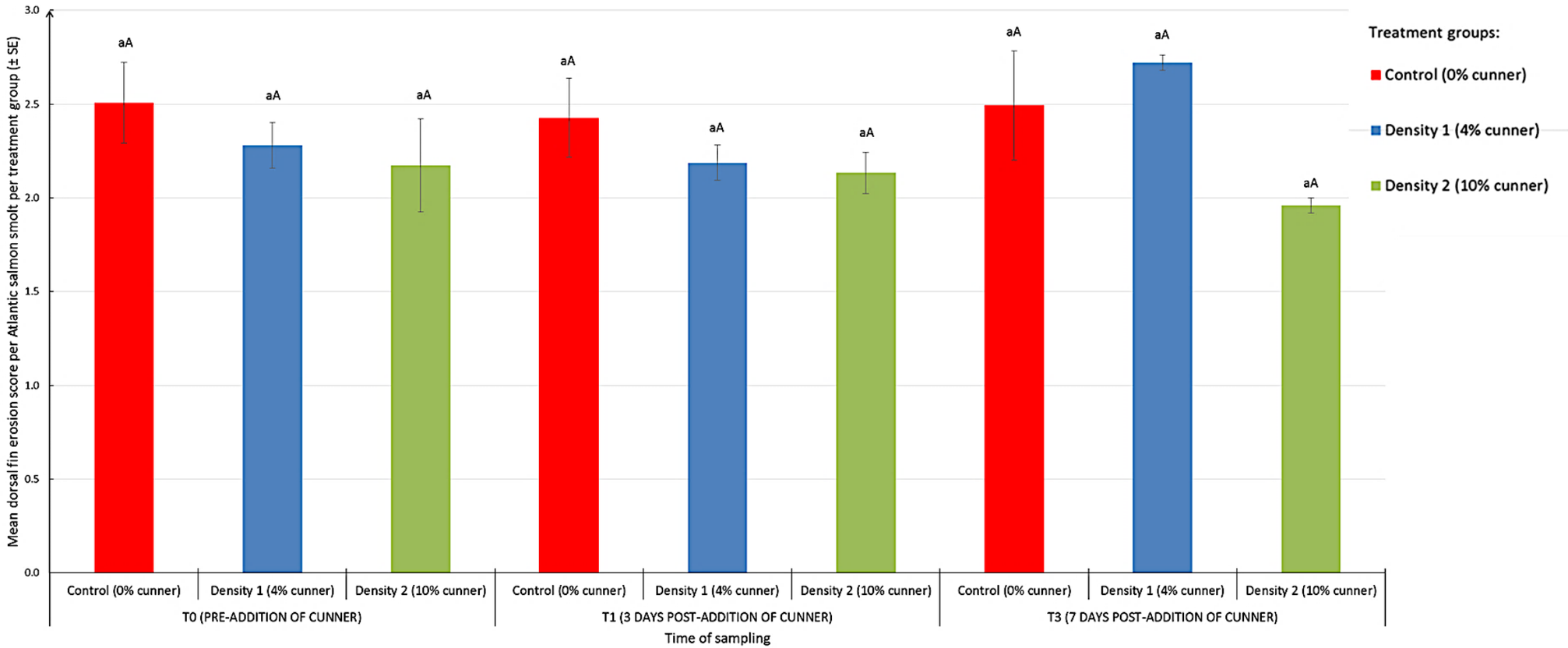

Figure 3-4. Mean dorsal fin erosion score of Atlantic salmon (*Salmo salar*) smolts sampled at 0, 3 and 7 days post-addition of cultured juvenile cunner (*Tautogolabrus adspersus*) stocked at densities of 0, 4 and 10% (treatment groups). Values represents mean dorsal fin erosion score per Atlantic salmon smolt per treatment group. Error bars represent plus and minus one standard error from the calculated mean. Lower case letters denote significance among treatment groups within the same time period. Upper case letters denote significance among treatment groups across sample periods.

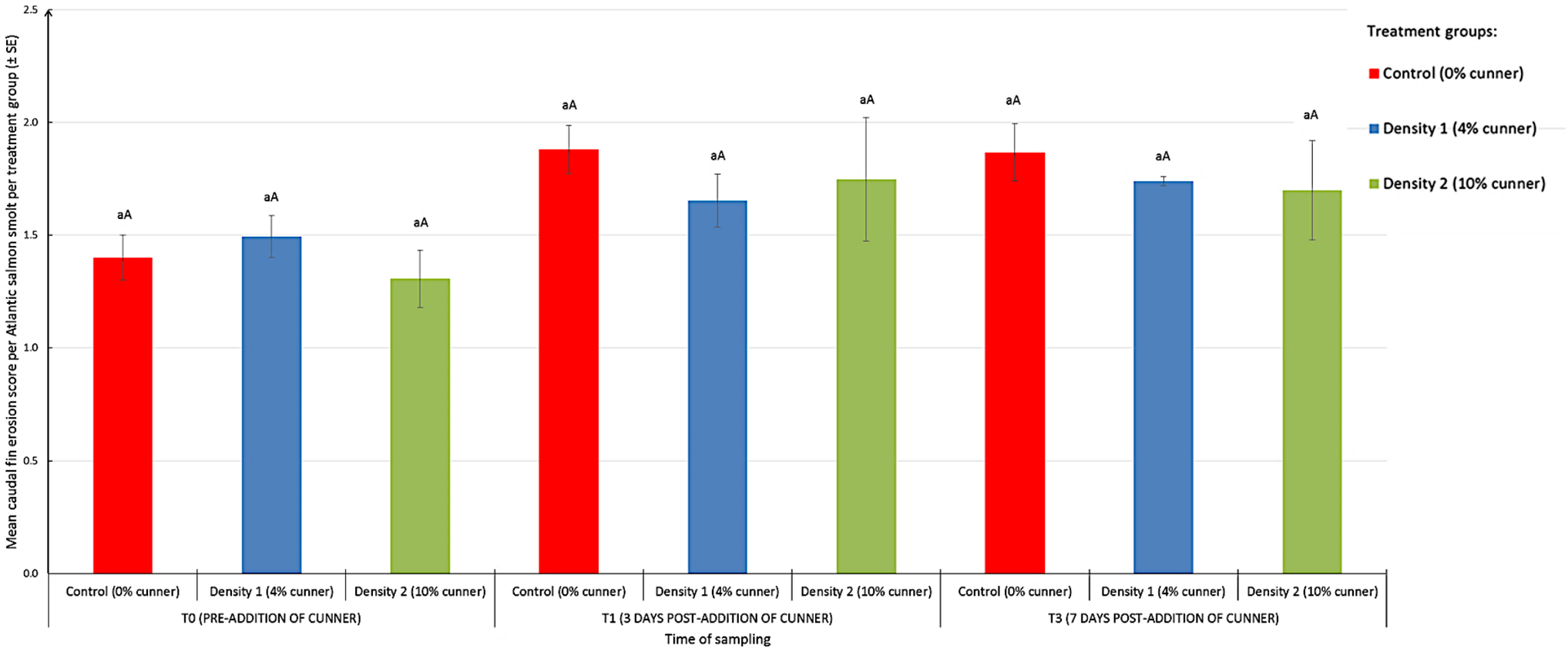

Figure 3-5. Mean caudal fin erosion score of Atlantic salmon (*Salmo salar*) smolts sampled at 0, 3 and 7 days post-addition of cultured juvenile cunner (*Tautogolabrus adspersus*) stocked at densities of 0, 4 and 10% (treatment groups). Values represents mean caudal fin erosion score of Atlantic salmon smolts per treatment group. Error bars represent plus and minus one standard error from the calculated mean. Lower case letters denote significance among treatment groups within the same time period. Upper case letters denote significance among treatment groups across sample periods.

3.5 Discussion

3.5.1 Delousing efficacy of cultured juvenile cunner

While the use of a tank-based system and an artificial sea lice parasitism model in this experiment afforded control over the sea lice abundance in each tank, the sea lice abundance on individual Atlantic salmon at the start of the experiment (prior to addition of cultured juvenile cunner) was difficult to control and individual fish variability occurred. To ensure that the sea lice attached to the Atlantic salmon smolts were the only food source available for cultured juvenile cunner, neither species was fed during the 7 days of cohabitation. Despite these efforts, both statistical results and qualitative observations suggested that the group of cultured juvenile cunners used in this study did not actively remove sea lice from Atlantic salmon smolts when cohabitated in tanks under laboratory conditions for a period of 7 days at stocking densities of 4 and 10% (Figure 3-2). There was an attempt to conduct in-tank video recordings of inter-species interactions, but this was not possible due to the difficulty associated with low light intensity and interior black colored tanks. As such, some interspecies interactions (e.g., cultured juvenile cunners occasionally approaching or swimming around Atlantic salmon smolts) were observed on an ad hoc basis throughout the experiment (this is not unexpected due to the fact that fish were not fed and were challenged with sea lice), but sea lice removal was not witnessed or confirmed through statistical analysis.

Unexpectedly, the mean sea lice number per Atlantic salmon smolt in all groups experienced a decrease from T0 to T1. It is unclearable as to what this may have been attributed to as the sampling protocol was consistant from T0 to T1, T1 to T2 and T2 to T3. Additionally, the Control group decreased significantly from T0 to T3 (18.3 ± 0.89 and 7.0 ± 0.51, respectively) (p=0.000) (Figure 3-2). This unexpected result could be attributable to sea lice becoming detached from Atlantic salmon smolts due to handling during each sampling event, which might explain why all three treatment groups (0, 4 and 10%) experienced a comparable decreasing mean sea lice number per Atlantic salmon

smolt from T0 to T3. This would not be unlike Glover et al. (2004) who noted that 3-3.6% of sea lice became detached from Atlantic salmon and were detected in the anesthetic bath during sampling for their laboratory-based experiment involving the handling of Atlantic salmon to conduct sea lice counts. Similarly, Nilsen et al. (2017) noted that under similar conditions of handling fish (i.e., conducting sea lice counts), there is an increasing possibility of sea lice detachment occurring due to crowding, dip netting and anesthesia. They suggested that in order to track sea lice detachment caused by fish handling during sampling events, any detached sea lice observed in sampling containers should be recorded. In the current study, it was not possible to avoid the handling of Atlantic salmon smolts as it was required to conduct sea lice counts and to assess fin condition. It is important to note that the sampling frequency of sea lice counts chosen for the current experiment was less than previously published research where the frequency of sampling was every 12 hours up to a period of 84 hours post-addition of farmed ballan wrasse to tanks containing Atlantic salmon (Leclercq et al., 2014). Due to logistitical constraints (e.g., time and labour), it was not possbile to count detached sea lice numbers in handling, anesthetic and recovery containers, to distinguish them from the count of attached sea lice for this experiment. Additionally, four samplings (T0-T3) occurred within 7 days with approximately 50% of Atlantic salmon smolts per tank being sampled each time, resulting in a high frequency of handling which may have resulted in an increased number of sea lice becoming detached. All tanks of fish were sampled and handled in a similar matter, therefore, the number of potentially dislodged lice should have been comparable, but this was not documented.

Individual PIT-tagged fish were not utilized for this experiement. This resulted in an inability to track individual fish across sampling periods and time (as they were not PIT-tagged), which may have contributed to the variability in sea lice numbers recorded for individual fish, tanks and treatments throughout the study. At each time of sampling, twenty-five Atlantic salmon smolts were systematically randomly selected from each tank

for sea lice counting and fin condition assessment. The limitation of sampling fish which were not individually PIT-tagged was the possibility that the twenty-five fish that were sampled at each time point were not the same twenty-five fish that were selected for subsequent samplings. This may have contributed to variability in the mean sea lice number on Atlantic salmon smolt per tank or by treatment group at each time of sampling. For future studies, using individually PIT-tagged experimental fish would increase the statistical power by allowing for repeated measurements on the same fish over time and minimizing potential individual variability between tanks. In order to increase the likelihood of a more similar initial sea lice parasitism level for each experimental fish or tank, instead of individually infecting Atlantic salmon smolt in each experimental tank (n=50 fish per tank), all experimental fish could be artificially infected with sea lice within one large tank. This could allow for the subsequent placement of Atlantic salmon smolts with more similar initial sea lice numbers into experimental tanks, prior to the addition of cleaner fish.

The inactive delousing by the cultured juvenile cunner used in this study may be attributed to several additional factors. For example, the potential effects of age and size of cultured juvenile cunners on their ability of to remove sea lice in this study is unknown; they may not have fully developed their delousing ability. As reported by Costa et al. (2016), cleaning behavior in wild-caught cunner is likely an opportunistic behaviour, one that does not occur naturally but may be acquired through cohabitation with salmon. However, Chao (1973) indicated that wild juvenile cunners feed on planktonic crustacea, therefore, it is plausible that cultured juvenile cunner may consume planktonic sea lice. Knowledge of the biology of wild cunner in captivity indicates that they become mature, in general, at a length of 8-11 cm. MacKinnon (1995) postulated that wild-caught juvenile cunners, which were less than 10 cm in length, were thought to be more inclined to graze on sea lice compared to the mature cunners (larger than 10 cm). Regarding the length and weight (10.5-12.5 cm and 20-25 g, respectively) of the cultured

juvenile cunners (approximately age 1+) employed in our experiment, they were thought to be approaching maturity, which may have resulted in an apparent disinterest in eating sea lice. Potential age- and size-related effects of cultured cunner on their ability to graze on sea lice would require further investigation.

It could be suggested that the food source available to cultured cunner under hatchery rearing conditions may impact the development of their delousing ability towards *L. salmonis*. The cultured juvenile cunner employed in the current study were the first generation progeny of wild-caught cunner broodstock, which were captured from the wild fishery and subsequently reared in a tank-based system under hatchery conditions. Wild cunner are omnivorous, eating a wide range of marine organisms as food sources in the open ocean (Chao, 1973). However, under artificial rearing conditions, cultured juvenile cunner are exposed only to commercial feed pellets, which could affect their interest in grazing on other food sources, such as sea lice.

The delousing ability of cunner could differ between individual fish. An early study investigating the potential of wild-caught cunner to remove *Caligus elongatus* from farmed Atlantic salmon under laboratory conditions determined that sea lice removal from individual salmon was not consistent, with some cunners effectively removing sea lice while others did not, suggesting that the delousing ability of cunner could vary individually, being based on individual cunner experiences and preferences rather than a typical “species-wide” behaviour (MacKinnon, 1995).

As suggested by Costa et al. (2016), the development of the delousing ability of wild-caught cunner may require a “learning curve”, so it could be assumed that cultured juvenile cunner may require the same. Canadian researchers (Costa et al., 2016) have recently documented potential cleaning behaviours (i.e., attempts of sea lice picking and chasing) by wild-caught cunner held in cohabitation with Atlantic salmon and suggested that wild-caught cunner might experience a “learning period” to develop delousing behaviours. Similarly, it has been suggested that a “learning component” is required for

cultured juvenile lumpfish, another cleaner fish species derived from wild-caught broodstock and cultured in hatcheries, which are being deployed in sea cages in Norway (Powell et al., 2018). This speculation was supported by the results of two field trials conducted in semi-commercial salmon farms. Through evaluation of the stomach contents of individual cultured juvenile lumpfish, as determined through gastric lavage, Imsland et al. (2014; 2015) found that the proportion of cultured juvenile lumpfish ingesting sea lice increased from 10% at Day 11 to 28% at Day 54 in one trial (2014) and from 13-17% at Day 11 to 33-38% at Day 77 in the other trial (2015).With regards to the difference in the time scale of tank-based laboratory trials versus field trials on salmon farms, the length of the trials vary. The time afforded cleaner fish for delousing in tank-based laboratory trials, in general, ranges from 24 hours to 7-10 days, which is thought to provide an adequate period for cleaner fish to exhibit delousing behaviour. In comparison, the length of time provided in field trials is usually longer, ranging from 1-2 weeks up to 1-2 months. Therefore, cleaner fish in field trials are afforded more time to develop or "learn" to prey on sea lice infested Atlantic salmon.

The absence of delousing ability of the cultured juvenile cunner employed in the current study could also be due to lack of conditioning to the cohabitation model prior to deployment. As previously mentioned, the cultured juvenile cunner used in the current study were fed with commercial pelleted feed as their only food source prior to the commencement of the experiment. Although they were acclimated to the same experimental conditions as the Atlantic salmon smolts, the cunner were not exposed to *L. salmonis* until they were introduced to the tanks of artificially infected salmon. This may have resulted in a limited amount of time for them to transition from eating commercial pelleted feed to sea lice. Furthermore, the 7-day experimental period may not have been sufficient for the cultured juvenile cunner to develop delousing behaviours. Affording the cultured juvenile cunner a longer acclimation and conditioning period with the sea lice infected Atlantic salmon prior to the first sampling event may have improved the study

design and subsequent results.

Another factor that might explain why cultured juvenile cunner did not actively remove sea lice was the lack of selection for individual cunner, or families of cunner, known to have well-developed delousing ability. Imsland et al. (2016) suggested that the delousing ability of cultured juvenile lumpfish could be genetically influenced or parentally controlled. They conducted a field trial investigating the sea lice removal and feeding preferences of nine different cultured juvenile lumpfish families' cohabitated with Atlantic salmon in sea pens. Out of nine lumpfish families, derived from wild-caught broodstock, five did not consume sea lice (*L. salmonis*) when cohabited with Atlantic salmon in sea cages over a period of 78 days. The remaining families demonstrated varying levels of sea lice consumption across the 78-day trial, raising speculation that the cleaning behaviour of cultured lumpfish towards *L. salmonis* may be variable and genetically related to their wild-caught parents. While not the focus of the current study, further investigation of individual cunner families with a well-developed ability to remove sea lice is likely warranted and such individuals would be desirable for use in breeding programs, where the individual fish or families which have well-developed sea lice delousing abilities could be selected as cleaner fish candidates for sea lice treatments or as broodstock in breeding programs.

3.5.2 Methods of assessing fin erosion as a fish welfare indicator

There is no a universal fin erosion scale or method adopted for the assessment of fish welfare, however, fin condition has been frequently used. As reviewed by Latremouille (2003), fin condition has previously been measured using a range of methods, including the use of fin condition indices as descriptive scales to describe either fin damage or erosion (Goede & Barton, 1990; Bosakowski & Wagner, 1994; Speare & MacNair, 1996; Turnbull et al., 1998; Moutou et al., 1998; MacLean et al., 2000; Turnbull et al., 2008). Bosakowski & Wagner (1994) devised a fin erosion scale, based

upon that of Goede & Barton's (1990), using a simplified 3-point scale to score fin erosion, where 0=perfect (no erosion), 1=slight erosion and 2=severe erosion. Developed from these ordinal indices, Speare & MacNair (1996) added an additional numerical estimation of fin absence and damage, where 0=normal; 1=up to 25% of fin missing or damaged; 2=25% to 75% of fin missing or damaged; 3=75% to 100% of fin missing or damaged; and 4=100% of fin missing or damaged and adjacent skin also affected. Another 4-point scale, derived from seven levels ofcatogorization of dorsal fin lesions of Turnbull et al. (1996), was used by Moutou et al. (1998) to classify fin damage as 0=no damage, 1=minor (<30% fin tissue loss), 2=severe (30-70% fin tissue loss) and 3=very severe (>70% fin tissue loss). MacLean et al. (2000) employed a 5-point scale where 0= >90% fin tissue undamaged, 1=60-90% fin tissue undamaged, 30-60% fin tissue undamaged, 10-30% fin tissue undamaged and <10% fin tissue undamaged. All these methods generally use a scoring system to describe fin condition and to quantify the degree of fin erosion. Subsequently, several photographic keys (Hoyle et al., 2007; Person-Le Ruyet et al., 2007) have been developed to provide assistance with the visual assessment of fin erosion, thus each assigned score corresponds to a particular photographic key. Such indices are advantageous in that they are simple and easy to use/adopt. It has been suggested that with the aid of photographic keys and descriptive scales, a trained examiner could assess each fin of a sampled fish within approximately 10 seconds, resulting in less handling and quick assessment, thus limiting the period of anaesthesia (Hoyle et al., 2007). Considering the intensive sampling schedule and sample size of the current study, a fin erosion score system with a photographic key was modified from the scales developed by Hoyle et al. (2007) (see Appendix D).

Fin damage (e.g., erosion and splitting) has been considered as one of the most common fish welfare indicators in aquaculture (Latremouille, 2003), particularly for Atlantic salmon (Turnbull et al., 1998; MacLean et al. 2000; Turnbull et al., 2008), as it is thought to be easy to assess. The mean fin erosion score at T0 (day prior to addition of

cunner) suggested that Atlantic salmon smolts employed in the current study had prior erosion of the dorsal and caudal fins, which is not an uncommon occurrence in hatchery-reared fish (Ellis et al., 2008). As shown in Figures 3-4, the mean dorsal fin erosion score of the three treatment groups ranged from 2.2 ± 0.25 to 2.5 ± 0.21 at T0, indicating that the erosion of the dorsal fins of the Atlantic salmon smolts were moderate to half eroded, prior to the addition of cultured cunner. The score for the caudal fin (Figure 3-5) was lower, ranging from 1.3 ± 0.13 to 1.5 ± 0.09, indicating that they were slightly and moderately eroded. It was anticipated that cultured juvenile cunner would perform delousing on Atlantic salmon smolts during cohabitation, which could increase potential interspecies interactions and potentially result in fin damage/erosion. If aggressive delousing behaviour occurred at densities of 4 and 10% cunner, the fin erosion scores for the dorsal and caudal fins would be expected to be higher at T1 and T3. However, the mean fin erosion score of dorsal and caudal fins of all three treatment groups remained constant from T1 to T3, suggesting the 7-day cohabitation period with cultured juvenile cunner did not impact the fin condition of Atlantic salmon smolts. These study findings necessitate reflection regarding the fin condition assessment method for fish originating from hatcheries (i.e., the fin erosion score for hatchery fish at T0 could start with a score which is greater than zero, where warranted).

It should be noted that limitations to the numerical quantification of fin erosion indices have been suggested. Latremouile (2003) noted two major limitations which include the fact that a fish with healthy fins, or a particular fin in perfect condition, are required as a baseline for comparison. This is not always possible and can be problematic, as was the case with the current study, where Atlantic salmon had visible fin erosion and damage prior to the addition of cunner (i.e., it likely originated at the hatchery). Moreover, although these indices use a combination of gross descriptions, percentages or photographic keys to categorize the degree of fin erosion, the assessment is subjective and may vary from individual to individual. Studies using these indices

could benefit from the assessment of the individual variability between scores assigned by multiple assessor. Hoyle et al. (2007) concurred that fin erosion score systems rely on the subjective interpretation of the examiner. To prevent the subjective assessment of such classification indices, Kindschi's (1987) fin condition factor was recommended by Latremouile (2003) and Hoyle et al. (2007). The calculation of fin factor, where, fin factor (%) = (fin length × 100) / (total fish length), allows for the quantification of the extent of fin erosion by measuring fin length (measured from the median point of attachment to the sampled fish's body, to the most distal median point on the selected fin) and fork length of the sampled fish, and is considered to be a more straightforward and accurate method. However, this method is time consuming and involves additional fish handling for the measurement of fin length, hindering its application in the current experiment and rendering it impractical for application on commercial farms for fish welfare evaluation. Although various scales and measurement methods have been applied to assess fin erosion as a fish welfare indicator, experimental objectives and analyses varied greatly between studies. Some studies focused on assessing erosion of selected fins (e.g., dorsal and pectoral fin, such as in Person-Le Ruyet et al., 2007), while others measured all fins and assigned scores for the individual fins (Person-Le Ruyet & Le Bayon, 2009). In more recent studies, scores of all fins were pooled and then averaged to represent an overall fin erosion as a fish welfare indicator (Arechavala-Lopez et al., 2013; Treasurer & Feledi, 2014).

Based on a survey of the literature, and the results of the current study, it is suggested that a 5-point fin erosion classification scale with corresponding photographic keys for each degree of fin erosion could be considered for future assessment of fish welfare when cleanerfish and Atlantic salmon are held in cohabitation. With respect to the current study, during the cleaner fish deployment period, individual Atlantic salmon smolt and cleaner fish species could be visually assessed with the modified 5-point scale to categorize the degree of fin erosion as a means to monitor potential changes in fish

welfare between the commencement and the end of the experiment. However, when cultured juvenile cunner and Atlantic salmon smolt were stocked at densities of 4 and 10% in the current study, no potential antagonistic interspecies interactions were witnessed. As such, it is difficult to say with certainty whether or not sufficient interaction occurred to allow for an accurate assessment of Atlantic salmon fin erosion.

There are several caveats to note with respect to the adoption of fin erosion as an assessment of fish welfare. It will be important to assess the fin erosion scores for a fish population prior to the deployment of cleaner fish in order to obtain a baseline fin erosion score. If this baseline score is not determined prior to the deployment of cleaner fish, in cases where baseline fin erosion is detected, subsequent changes in fin erosion could be attributed to the effect of cohabitation with the cleaner fish, when in fact they are not. In addition, an individual who assesses fin welfare must be properly trained to use the chosen fin erosion scales and methods. In order to ensure a consistent scoring method, either the same examiner should be designated to conduct all fish welfare assessments throughout the experiment using the adopted fin erosion score scale, or multiple examiners could assess the same fish using the adopted fin erosion scale and their assessments could subsequently be compared for agreement/correlation. Otherwise, individual fish sampled by multiple examiners at the same time could result in differences or biases between individual examiners.

While the length of the cohabitation period for the current study may not have been sufficient (as discussed above), the cohabitation model employed was suitable as a means by which to assess any potential damage to the dorsal and caudal fins of Atlantic salmon smolts attributable to potential interspecies interactions. However, if the sea lice burden is too high in these types of experiments, fin erosion could also be attributable to damage caused by the sea lice infestation (González et al., 2020).

3.6 Conclusion

Although some inter-species interactions were observed during the course of this experiment, the cultured juvenile cunner employed in this study did not exhibit delousing behavior when stocked at densities of 4 and 10% during 7 days of cohabitation with farmed Atlantic salmon, nor did their presence result in a reduction in sea lice counts. While the dorsal and caudal fin scores did not change during the 7 days of cohabitation, it was difficult to say with certainty whether or not sufficient interaction occurred to allow for an accurate assessment of Atlantic salmon fin erosion. Future recommendations include holding both species in cohabitation for a longer acclimation period, as the 7 days of cohabitation applied in this experiment may have been too short for cultured juvenile cunner to learn or develop any delousing behaviours and to engage in interspecies interactions. Moreover, if conditions permit, the stomach contents of cultured juvenile cunner could be examined for evidence and confirmation of sea lice grazing. A breeding program could be developed to select for progeny which are derived from known lice-eating cunner parents or families prior to deployment in sea cages for large-scale application.

3.7 References

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CHAPTER 4. An evaluation of the effect of water temperature on delousing efficacy of cultured juvenile cunner (*Tautogolabrus adspersus*) against *Lepeophtheirus salmonis* artificially infecting Atlantic salmon (*Salmo salar*) smolts and prevalence of pathogens in both species during cohabitation

4.1 Abstract

This laboratory study investigated the effect of water temperature on the delousing efficacy of cultured juvenile cunner when cohabitated with Atlantic salmon smolts artificially infected with *L. salmonis* and the prevalence of commercially significant pathogens and Reportable Diseases (e.g., *Renibacterium salmoninarum* causing Bacterial Kidney Disease (BKD), Infectious Pancreatic Necrosis virus (IPNv), Infectious Salmon Anaemia virus (ISAv), Viral Hemorrhagic Septicemia virus (VHSv), Infectious Haematopoietic Necrosis virus (IHNv), Nodavirus, Vibrio spp. and *Aeromonas salmonicida*) in Atlantic salmon smolts and cultured juvenile cunner following 7 days of cohabitation. Fish were artificially infected with sea lice and subsequently held at 18°C (high water temperature group; HWT) or 2°C (low water temperature group; LWT) under laboratory conditions. Each temperature group had a control treatment (0% cunner) and an experimental density treatment (10% cunner) and was comprised of three experimental tanks per treatment. The delousing efficacy of cultured juvenile cunner was investigated by conducting sea lice counts on Atlantic salmon smolts at three time points: the day prior to the addition of cunner (T=0); 3 days post-addition of cunner (T=1); and 7 days post-addition of cunner (T=2). Neither High or Low water temperature or the presence of cunner had a significant influence on the mean sea lice per Atlantic salmon smolt per tank amongst the four treatment groups over three sampling periods (p=0.093),

suggesting that the group of cultured cunner employed in the current study did not actively remove *L. salmonis* during 7 days of cohabitation. Regarding the prevalence of commercially significant pathogens in Atlantic salmon smolts and cultured juvenile cunner held in cohabitation, there were no detections of *Renibacterium salmoninarum* causing BKD, IPNv, ISAv, VHSv, IHNv, Nodavirus, Vibrio spp., *Aeromonas salmonicida* or any other pathogen that may have been detected by these tests. These results suggest that the research population of cultured juvenile cunner and Atlantic salmon were neither detected with any pathogens nor obsevered with clinical signs of dieases during a 7-day period of cohabitation.

4.2 Introduction

Cultured cunner (*Tautogolabrus adspersus*) are currently being investigated for use in commercial Atlantic salmon (*Salmo salar*) sea cages sites in Atlantic Canada for the control of sea lice (*Lepeophtheirus salmonis*) (Boyce et al., 2018). To date, limited knowledge is available regarding the effect of water temperature on the delousing performance of cultured juvenile cunner and the prevalence of commercially relevant pathogens of cultured juvenile cunner and Atlantic salmon when held in cohabitation.

Among various factors in the aquatic environment, water temperature plays an important role in poikilothermic fish (Newell, 1966; Valerio et al., 1992), including

cleaner fish, impacting their movement, feeding, growth and metabolism (Sayer & Reader, 1996; Nytro et al., 2014; Moran et al., 2019; Yuen et al., 2019). In North America, wild cunner (*Tautogolabrus adspersus*) were found to exhibit a state of torpor (i.e., physical inactivity, lethargy) in response to low seawater temperatures in several laboratory trials (Haugaard & Irving, 1943) and through field observations (Green & Farwell, 1971; Dew, 1976; Pottle & Green, 1979). Early laboratory studies aimed at investigating the metabolism of cunner in response to water temperature determined that wild cunner (25-70 g) experienced a metabolic depression (a decrease in oxygen consumption) at temperatures below 5°C (Haugaard & Irving, 1943). Similarly, Chao (1973) observed decreased feeding activity in three size groups of wild cunners (30-50 mm, 100-225 mm and 230-300 mm) when the water temperature in aquaria housed under laboratory conditions was decreased below 4-6°C. In the waters off Newfoundland, early field observations documented cunner experiencing a state of torpor (e.g., lack of physical activity and feeding) when water temperatures decreased below 5°C; a temperature that occurs mostly during the 5-6 months of the winter season (Costa et al., 2013; Kelly et al., 2014). In recent years, additional behavioural and physiological studies have been conducted on wild-caught cunner in order to investigate the depression of metabolism behind torpor or winter dormancy at low water temperatures (Costa et al., 2013; Kelly et al., 2014). This metabolic response could potentially prevent cunner from

being active and exhibiting delousing performance at temperatures below 5°C (Boyce et al., 2018). While ambient surface seawater temperatures can reach 18-20ºC in Newfoundland in the summer months (Boyce et al., 2018), and as high as 24ºC in the Bay of Fundy, New Brunswick (Brewer-Dalton et al., 2015), there is limited research related to the potential effects of high water temperature on cultured cunner delousing ability.

The North American cunner behaviour, described above, is similar to that of wild-caught wrasse (e.g., goldsinny wrasse) in Europe when they are exposed to low water temperatures (Costa et al., 2013; Kelly et al., 2014). Darwall et al. (1992) reported field observations made by SCUBA divers which noted a decrease in the numbers of active goldsinny wrasse in Irish waters below 7ºC. Similar field observations of various wrasse species (i.e., goldsinny, rock cook, ballan and cuckoo wrasse) made on the west coast of Scotland (Sayer et al., 1993; 1994) noted some species (e.g., goldsinny, rock cook and ballan wrasse) exhibited a torpid state during winter months in water temperatures of approximately 6ºC (Treasurer, 1993). This temperature-dependent behaviour of wrasse (e.g., goldsinny, rock cook and corkwing wrasse) has been verified through a series of laboratory experiments on survival, physiological (e.g., blood physiology, oxygen uptake) and metabolic parameters (e.g., opercular motion and heart rate) via artificially exposing wrasse to various water temperatures ranging from 4-10ºC (Sayer & Reader, 1996; Sayer & Davenport, 1996; Sayer, Reader & Davenport, 1996). In addition, marked declines in

cleaning activity of wild-caught goldsinny and corkwing wrasse were observed by SCUBA divers in October, 1992 when water temperature dropped from 13 to 11ºC in a commercial cleaner fish trial carried out on an Irish Atlantic salmon farm (Deady et al., 1995). These findings postulate that the behaviour of certain wrasse species tends to be impacted by low water temperatures, and the commencement of torpor and winter dormancy occurs when seawater temperatures drop below 6ºC.

When held in cohabitation with Atlantic salmon in commercial sea cages, cleaner fish are exposed to diverse marine environmental conditions, which includes, but is not limited to; changing seawater temperatures. Water temperature has been shown to impact the ability of various wild-caught and cultured cleaner fish species to remove sea lice from cultured Atlantic salmon, such that metabolic depression caused by sub-optimal environmental or rearing temperatures has been shown to suppress general feeding activities, which, in turn, compromises their delousing efficacy (Sayer, Reader & Davenport, 1996; Imsland et al., 2014; Powell et al. 2018).

As a new cleaner fish candidate in Atlantic Canada, the effect of water temperature on cultured cunner delousing efficacy has not been thoroughly investigated. However, information related to other cleaner fish candidate species is better understood. For example, in recent years, hatchery reared lumpfish (*Cyclopterus lumpus*), derived from wild-caught broodstock, have been identified as a cold-water alternative to the

various wrasse species that are known to lose activity at lower water temperatures (Imsland et al., 2014). It has been demonstrated under laboratory conditions that cultured juvenile lumpfish actively feed and grow at a temperate as low as 4ºC, thus having the potential to survive winter water temperatures in net pens with Atlantic salmon (Nytrø et al., 2014). In a field trial conducted over several winter months, 87 out of 90 (97%) of cultured lumpfish of three size groups (22.6 ± 0.7 g, 77.4 ± 3.6 g and 113.5 ± 2.1 g) survived and demonstrated the ability to remove sea lice (*L. salmonis*), which was reflected by the decrease in sea lice counts when the water temperature in the sea cages was as low as 4.5ºC (Imsland et al., 2016).

Disease transmission from cleaner fish species to Atlantic salmon during cohabitation in sea cages has been suggested (Treasurer, 2012; Powell et al., 2018; Scholz et al., 2018).Whether or not cleaner fish (i.e., wild-caught and cultured) could act as potential vectors for a range of bacterial and viral diseases under different water temperatures is currently unknown. As speculated by Treasurer (2012), the occurrence of clinical furunculosis was thought to be attributed to a high water temperature condition while holding and transporting wild-caught wrasse to salmon farms. While no published reports have emerged to date providing evidence of disease transmission from cleaner fish species to Atlantic salmon under farm conditions, new diseases (e.g., bacterial and viral) have been identified and reported in wild-caught and cultured cleaner fish species

in Europe (Treasurer, 2012; Scholz et al., 2018). In Canada, there are no confirmed reportable diseases (CFIA, n.d.) in cunner (Boyce et al., 2018). The development and implementation of a cleaner fish health surveillance program would aid in the detection of diseases through routine disease surveillance and regular health screening of live fish and mortalities.

The objectives of this experiment were: (1) to investigate the effects of high and low water temperature on the delousing efficacy of cultured juvenile cunner on Atlantic salmon smolts artificially infected with *L. salmonis*; and (2) to investigate the prevalence of pathogens in Atlantic salmon smolts and cultured juvenile cunners held in cohabitation. To the authors' knowledge, this is the first laboratory study that investigates the effect of water temperature on cultured juvenile cunner delousing efficacy under laboratory conditions and the pathogen prevalence of economically important viral and bacterial pathogens in Atlantic salmon smolts and cultured juvenile cunner when held in cohabitation.

4.3 Materials and Methods

4.3.1 Laboratory rearing tank system

This experiment was conducted in the same saltwater recirculating aquaculture system (RAS) as described in Experiment 1 (Chapter 3). The system used contained twelve 1.5 cubic meter tanks (1500 L), each containing 700 L of saltwater (31-33 ppt),

initially maintained at 11 ± 1ºC with individual tank level control through the use of a typical “stand pipe” approach. Each stand pipe was connected to a 4” effluent line that never exceeded 50% capacity. The effluent lines flow to a vortex separatos where approximately 90% of solids are removed followed by additional water polishing in Hydrotech Drum Filters containing 19 micron filter screens. This system removes all particles greater than 19 microns and ensures no cross-tank contamination. Rearing conditions (e.g., photoperiod, dissolved oxygen levels, salinity, pH, etc.) were identical to Experiment 1 (Chapter 3, Section 3.3.1). All water quality parameters remained in the normal range for Atlantic salmon smolts throughout the course of the experiment. To maintain experimental tanks at two separate system temperatures, a total of 12 experimental tanks were divided into two banks of 6 tanks, each supplied with a separate water inflow. The water temperature of each bank of tanks was independently controlled by a heating/cooling system which was used to adjust the water temperature for the temperature treatment groups (i.e., HWT, 18ºC and LWT, 2ºC). There were two additional tanks in the RAS system for temporarily housing the cultured juvenile cunners prior to the start of the experiment. Each of these tanks contained artificial “hides”.

4.3.2 Experimental fish

The animals were handled and cared for in accordance with the Canadian Council

on Animal Care's Guidelines on the Care and Use of Fish in Research, Teaching and Testing (Canadian Council on Animal Care, 2005). The study was approved by the Animal Care Committee of Memorial University (ACP 15-02-JW).

Atlantic salmon smolts (n=600, 331.7 ± 3.7 g, 30.9 ± 0.1 cm, Saint John River strain), which were obtained from a commercial Atlantic salmon hatchery in Newfoundland and Labrador, were equally allocated amongst twelve experimental tanks (n=50 per tank). The experimental tanks were randomly assigned to one of four treatment groups (three tanks per treatment group). Atlantic salmon were hand-fed to satiation twice daily with Corey® Marine Aquafeed (4mm) prior to the addition of cunners. The daily feed consumption per tank was recorded (although feed consumption data has not been presented, there was no indication of differences in feed consumption between tanks). Feeding was stopped when cunner were added to experimental tanks containing Atlantic salmon. Cultured juvenile cunners (within a size range of 20-25 g in body weight and 10-12 cm in fork length) were supplied by JBARB, and temporarily housed under the same rearing conditions in two separate tanks within the same RAS system containing the twelve experimental tanks. The cultured juvenile cunners used in this experiment were the first generation progeny (F1 stock) of wild-caught broodstock, chosen from the same population as those used in Experiment 1 (Chapter 3). This group of cultured juvenile cunners were hand-fed to satiation every two days prior to cohabitation with Atlantic

salmon, using a mixture of two types of marine species diets: Skretting® North America Gemma Diamond (pellet size: 1.8 mm, Lot # 7220772) and Europa® (pellet size: 2.0 mm, Lot # 8601A). Neither species was fed during the 7 day cohabitation period.

4.3.3 Artificial sea lice parasitism

The artificial sea lice parasitism followed the same procedures as described in Chapter 3 (Section 3.3.3) with the following exceptions. All twelve experimental tanks of Atlantic salmon smolts were artificially infected with approximately 20-25 *L. salmonis* copepodids per fish on Oct. 3rd, 2016. Due to the fact that the number of infectious copepodids available for this experiment was limited (due to a limited number of sea lice egg strings collected in the field), there was consequently no second parasitism conducted. A 3000 mL subsample of saltwater containing approximately 1000-1200 *L. salmonis* copepodids were added to each experimental tank (Figure 4-1).

4.3.4 Experimental design

A random draw was used to ensure each tank had an equal probability of being assigned to each treatment group. The twelve experimental tanks were randomly assigned to one of four treatment groups which were classified according to temperature and the stocking density of cultured juvenile cunner. Fish held at 18°C were labeled as the high

water temperature group (HWT) and those held at 2°C were labelled as the low water temperature group (LWT). Each temperature group had a control treatment (0% cunner) and an experimental density treatment (10% cunner). Each treatment group was comprised of three replicate tanks.

4.3.5 Water temperature adjustment

Approximately 3 weeks following the artificial sea lice parasitism, the attached copepodids on Atlantic salmon smolts subsequently developed to mobile stages (i.e., pre-adult and adult). Starting on Oct. 26th, 2016 (23 dpi), 13 days prior to the addition of the cultured cunner, the water temperature of the LWT treatment groups was gradually decreased from 11 ± 1ºC to 2 ± 0.4ºC (over a period of approximately 8 days). For the HWT treatment groups, the water temperature was gradually increased from 11 (± 1ºC) to 18ºC (± 0.3ºC) starting on Oct. 30th, 2016 (27 dpi) (over a period of approximately 5 days). Once the desired water temperatures (2ºC & 18ºC) were achieved, the experimental fish (i.e., Atlantic salmon smolts and cultured juvenile cunners) in both the HWT and LWT treatment groups were provided 3-4 additional days to acclimate to the new water temperatures (Figure 4-1).

4.3.6 Fish sampling

4.3.6.1 Sea lice counts

On Nov. 7th, 2016 (35 dpi) (the day prior to the addition of cunner; T=0), all fifty Atlantic salmon smolts in each tank were anaesthetized using of tricaine methanesulphonate (4 mg/L TMS; MS-222), body weight (g) and fork length (cm) were measured and sea lice counts (pre-adult and adult stages) were recorded. The following day, five cultured juvenile cunners within a size range of 20-25 g in body weight and 10-12 cm in fork length were randomly added to tanks in the HWT (10% cunner) and LWT (10% cunner) treatment groups. Two additional sea lice counts were conducted on up to 50 fish (the number of fish sampled in some tanks may have been up to 5 fewer fish due to mortalities) from each experimental tank 3 days (39 dpi) (T1) and 7 days (43 dpi) (T2) post-addition of cultured juvenile cunner (Figure 4-1).

4.3.6.2 Sampling for pathogen prevalence

Non-probability sampling (divided as equally as possible between tanks) was used to obtain an additional 30 Atlantic salmon smolts and 21 cultured juvenile cunner from the experimental population were sampled for pathogen prevalence testing on Oct. 27th and Oct. 31st, 2016, respectively. At the conclusion of the experiment, non-probability sampling (divided as equally as possible between tanks) was used to obtain a total of 36

Atlantic salmon smolts (i.e., 3 fish per tank from each of the 12 experimental tanks) and all 30 cultured juvenile cunners which were cohabitated with Atlantic salmon smolts were sampled on Nov. 15th and Nov. 16th, 2016, respectively (Figure 4-1). A necropsy was performed on each animal and brain, eye, heart, spleen, liver, pyloric cecea, kidney and gill tissues were collected for histology. Brain, eye, heart, spleen, kidney and gill tissues were collected for viral culture on Atlantic salmon kidney (ASK), Chinook Salmon Embryo (CHSE), Epithelioma Papulosum Cyprini (EPC) and E11 cell lines. VHS (heart and kidney), ISA (gill and kidney) and Nodavirus (brain and eye) were tested using PCR. Indirect fluorescent antibody test (IFAT) was used for the detection of Renibacterium and ISA from kidney imprints. Eye, heart and kidney tissues were sampled for bacteria of concern using Selective Kidney Disease Medium (SKDM), Trypticase Soy Agar (TSA), Blood Agar (BA) and Marine Agar (MA). Samples were submitted to accredited laboratories (Atlantic Veterinary College, University of Price Edward Island, Canada and Research and Productivity Council, New Brunswich, Canada) for diagnostic testing. Atlantic salmon samples were subjected to an indirect fluorescent antibody test (IFAT) for the bacteria that causes bacterial kidney disease (BKD). Reverse transcription polymerase chain reaction (RT-PCR) was employed for the detection of Infectious Pancreatic Necrosis virus (IPNv), Infectious Salmon Anaemia virus (ISAv), Viral Hemorrhagic Septicemia virus (VHSv), Infectious Haematopoietic Necrosis virus (IHNv)

and Nodavirus. Cultured juvenile cunner samples were tested for Nodavirus using RT-PCR.

4.3.6.3 Examination of cultured juvenile cunner digestive tracts

The digestive tracts of 30 cultured juvenile cunners (the same fish that were sampled for pathogen prevalence testing at the end of the experiment as described above) were examined at the end of the experiment for the presence of ingested sea lice.

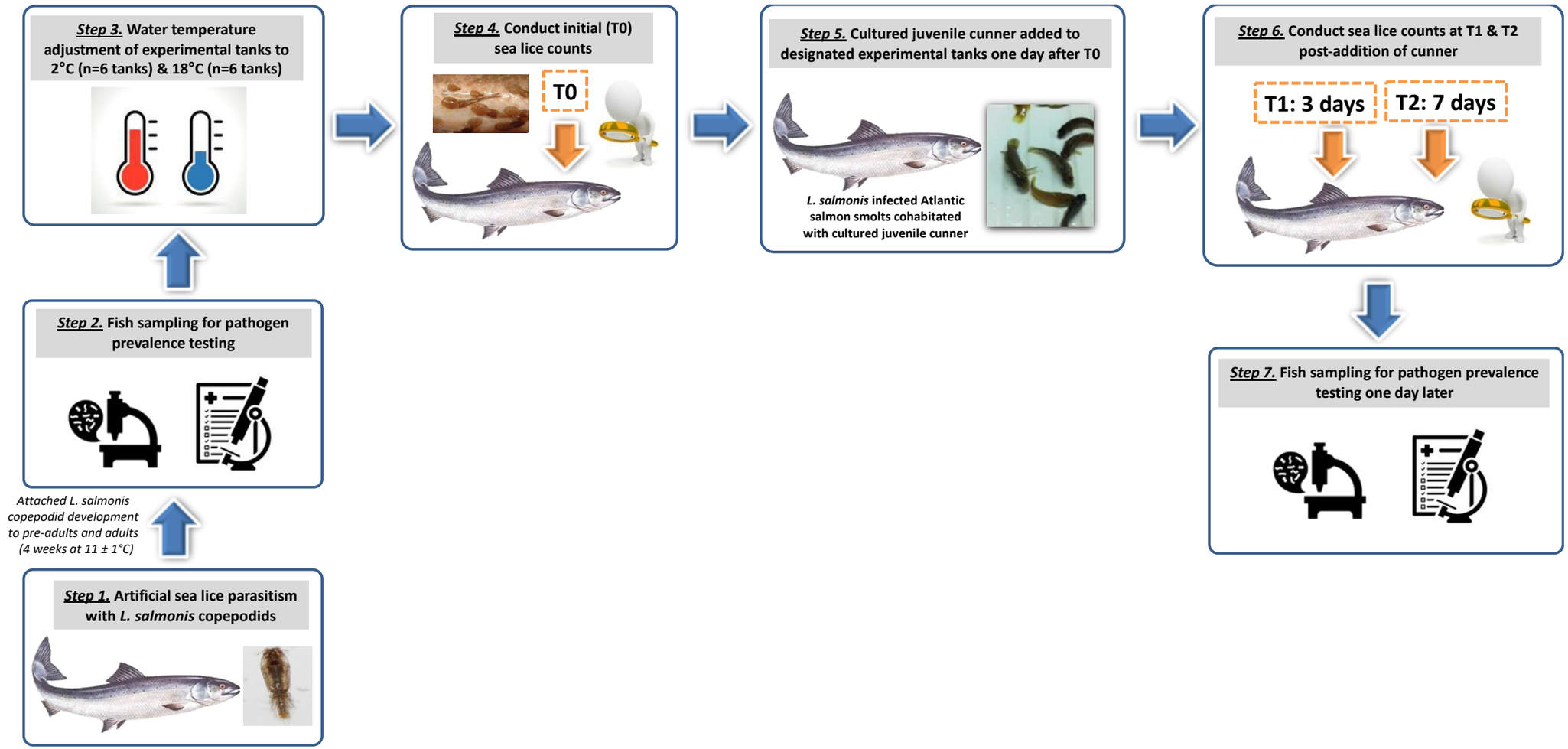

Figure 4-1. Experimental design and procedures.

4.3.7 Statistical analysis

Statistical analyses were conducted using STATA/SE™ 15 (special edition) statistical software (StataCorp, 2017). The effect of treatment, time of sampling and tank on the mean sea lice number per Atlantic salmon smolt per tank sampled at 0, 3 and 7 days post-addition of cultured juvenile cunner was examined using a liner regression model. The mean sea lice number per Atlantic salmon smolt per treatment group was assessed by one-way analysis of variance (ANOVA) and the significance between treatment groups within the same time period and the significance between treatment groups across sample periods was compared using a Tukey's multiple comparison test (Minitab® 17). Statistical differences were considered significant at $p<0.05$. Based on the results on the normality test, only one standard error of the mean is displayed in each figure.

4.4 Results

Based on the results of the regression analysis (which included variables for treatment, time and tank), there was no statistically significant effect of treatment ($p=0.093$), time ($p=0.333$) or tank ($p=0.142$) on the mean sea lice number per Atlantic salmon smolt when held at 18°C (HWT) and 2°C (LWT) at densities of 0 and 10% cunner for seven days (Figure 4-2). Although not attributable to a treatment effect, the

mean sea lice number per Atlantic salmon smolt in the HWT treatment groups (i.e., HWT, 10% cunner and HWT Ctrl., 0% cunner) decreased significantly from T0 (pre-addition of cunner) to T2 (7 days post-addition of cunner) based on the results of Tukey's multiple comparison test. However, the mean sea lice number per Atlantic salmon smolt for the LWT treatment groups (i.e., LWT, 10% cunner and LWT Ctrl., 0% cunner) remained unchanged within this time period (Figure 4-2).

When the HWT and LWT treatment groups were compared separately, there was no significant effect of the presence (10% cunner) or absence (0% cunner) of culture juvenile cunners in tanks on the mean sea lice number per Atlantic salmon smolt when held at water temperature of 18ºC (HWT) (p=0.200) and 2ºC (LWT) (p=0.503) over a 7 day cohabitation period.

The results of this experiment suggest that the group of cultured juvenile cunners tested in this laboratory experiment did not remove *L. salmonis* from Atlantic salmon smolts at high (18ºC) or low (2ºC) water temperatures. This was supported by the fact that no sea lice or any other fragments of digested sea lice were observed in their digestive tracts when examined at the end of the experiment.

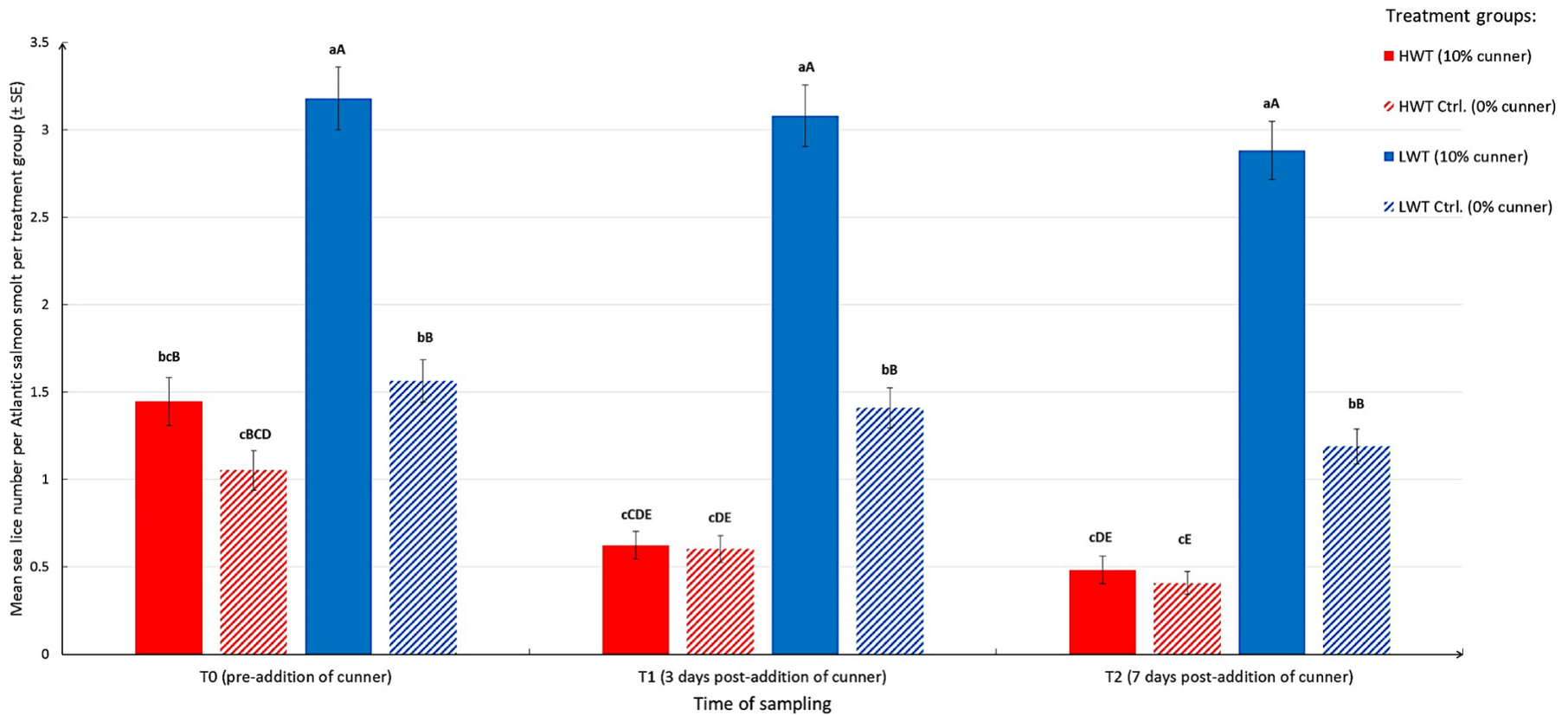

Figure 4-2. Mean sea lice (*Lepeophtheirus salmonis*) number on Atlantic salmon (*Salmo salar*) smolts sampled at 0, 3 and 7 days post-addition of cultured juvenile cunner (*Tautogolabrus adspersus*) held in water temperatures of 18°C (HWT) and 2°C (LWT), respectively, at densities of 0 and 10% cunner. Values represent mean sea lice number per Atlantic salmon smolt per treatment group. Error bars represent plus and minus one standard error from the calculated mean. Means that do not share the same letter are significantly different. Lower case letters denote significance among treatment groups within the same time period. Upper case letters denote significance among treatment groups across sample periods.

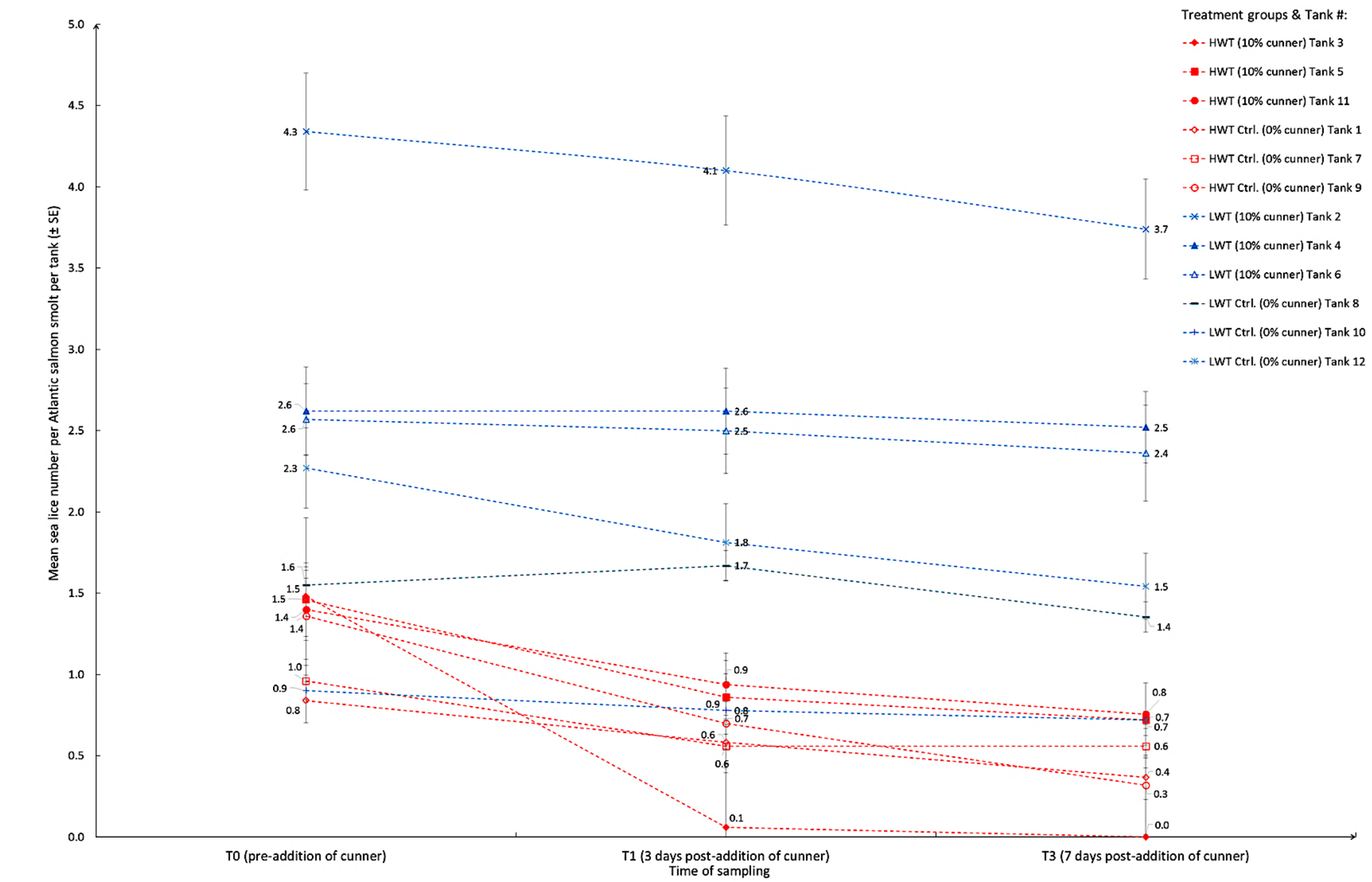

Figure 4-3. Tank level mean sea lice (*Lepeophtheirus salmonis*) number on Atlantic salmon (*Salmo salar*) smolts in each experimental tank sampled at 0, 3 and 7 days post-addition of cultured juvenile cunner (*Tautogolabrus adspersus*) held in water temperatures of 18°C (HWT) and 2°C (LWT) at densities of 0 and 10% cunner, respectively.

For all Atlantic salmon smolt and cultured juvenile cunner tissue samples sampled prior to and at the end of the 7 day cohabitation, no abnormal findings were detected at necropsy (Table 4-1). Results from the diagnostic laboratories indicate that, histologically, no significant abnormalities were detected and all findings were within the normal range for the age and species of fish. In Atlantic salmon sampled on Nov 15th, 2016 when the experiment was complete, most fish samples showed a higher accumulation of melanomacrophages in kidney than expected normal levels, which is often associated with either malnutrition/wasting or higher than normally expected tissue turnover (e.g., recovering from an infection, recent vaccination, recent exposrure to a pathogen/toxin or a result of poor nutrtion). No microbial growth was detected for eye, heart and kidney samples from either species. The indirect fluorescent antibody test (IFAT) for detecting *Renibacterium salmoninarum* for all sampled fish was negative. No viruses were isolated from viral cultures for any of the tissue samples. Atlantic salmon sampled on Oct. 27th and Nov 15th, 2016 tested negative for IPNv, ISAv, VHSv, IHNv, and Nodavirus. The cultured juvenile cunner tissue samples collected on Oct. 31st and Nov. 16th, 2016 also tested negative for Nodavirus.

Table 4-1. Disease surveillance test results for Atlantic salmon (*Salmo salar*) smolts and cultured juvenile cunner (*Tautogolabrus adspersus*) sampled prior to cohabitation and at study termination after 7 days cohabitation in a saltwater Recirculating Aquaculture System.

SAMPLING DATE	Oct. 27, 2016	Oct. 31, 2016	Nov. 15, 2016	Nov. 16, 2016
SPECIES	Atlantic salmon	Cunner	Atlantic salmon	Cunner
NUMBER OF SAMPLES	30	21	36	30
NECROPSY FINDINGS	No abnormal findings detected	No abnormal findings detected	No abnormal findings detected	No abnormal findings detected
HISTOPATHOLOGY (brain, eye, heart, spleen, kidney and gill tissues)	• No significant abnormalities detected • All findings within the normal range for that age and species	• No significant abnormalities detected • All findings within the normal range for that age and species	• No significant abnormalities detected • All findings within the normal range for that age and species • Histologically most fish showed a higher than expected accumulation of melanomacrophages in the kidney associated with either malnutrition/wasting or higher than normally expected tissue turnover	• No significant abnormalities detected • All findings within the normal range for that age and species
BACTERIOLOGY (eye, heart, kidney)	No microbial growth	No microbial growth	No microbial growth	No microbial growth
INDIRECT FLUORESCENT ANTIBODY TESTING (IFAT) FOR BKD[a] (kidney)	Negative	Negative	Negative	Negative
VIRAL CULTURE (brain, eye, heart, spleen, kidney and gill tissues)	No virus isolated	No virus isolated	No virus isolated	No virus isolated
RT-PCR[f] (VHS (heart and kidney), ISA (gill and kidney) and Nodavirus (brain and eye))	Negative for IPNv[b], ISAv[c], VHSv[d], IHNv[e], Nodavirus	Negative for IPNv[b], ISAv[c], VHSv[d], IHNv[e], Nodavirus	Negative for IPNv[b], ISAv[c], VHSv[d], IHNv[e], Nodavirus	Negative for IPNv[b], ISAv[c], VHSv[d], IHNv[e], Nodavirus
ADDITIONAL COMMENTS	No significant abnormalities detected in this population	No significant abnormalities detected in this population	No significant abnormalities detected in this population	No significant abnormalities detected in this population

[a] Bacterial Kidney Disease

[b] Infectious Pancreatic Necrosis virus

[c] Infectious Salmon Anaemia virus

[d] Viral Hemorrhagic Septicemia virus

[e] Infectious Haematopoietic Necrosis virus

[f] Reverse transcription-polymerase chain reaction

4.5 Discussion

The results of the current study suggest that the group of cultured juvenile cunners tested in this laboratory experiment did not remove *L. salmonis* from Atlantic salmon smolts at high (18ºC) or low (2ºC) water temperatures over a 7 day period. One potential reason for the lack of a statistically significant finding could be the fact that sea lice counts in the HWT groups were low (0 to approximately 1.5 lice per fish, see Figure 4-2). Another potential explanation for the lack of delousing activity by cultured juvenile cunner is that their potential delousing ability may have been hindered at the high and low water temperatures selected for the experiment (i.e., 18 and 2ºC), and the length of the acclimation period to these temperature was likely not sufficient. With regards to the early findings related to the general biology of cunner, the feeding behaviour/activity of cunner is likely temperature-independent. Wild cunner (*Tautogolabrus adspersus*) inhabit shallow and inshore marine environments in the waters of the western Atlantic, ranging from Newfoundland and the Gulf of St. Lawrence in Canada to Chesapeake Bay in the USA (Johansen, 1925; Scott & Scott, 1988; FishBase, n.d.). Along their coastal distribution of habitats, especially in waters off Atlantic Canada, Atlantic salmon aquaculture activities occur in the coast of bays region of the south coast of Newfoundland and in the Maritime Region, primarily the Bay of Fundy, New Brunswick. These areas experience seasonal variances in surface sea water temperatures that vary by

location in relation to various oceanographic and geographic conditions (Brewer-Dalton et al., 2015). In summer, ambient surface seawater temperature can reach 18-20ºC in Newfoundland and 24ºC in the Bay of Fundy, NB (although not likely at a depth where Atlantic salmon are farmed); in the winter temperatures can decrease to 0ºC and below the freezing point of seawater (-1.9ºC), and in some extreme cases, even colder at -2.3ºC (Brewer-Dalton et al., 2015; Boyce et al., 2018). As cunner experience a state of torpor at temperatures below 4-6ºC (Green & Farwell, 1971), it is hypothesized that this could potentially hinder their delousing ability or even impact their survival at low water temperatures. The ability of cultured cunner to cope with seasonal fluctuations in water temperature, including extreme low temperatures (below 0ºC) or relatively high water temperatures in summer (e.g., 18-24ºC), is currently unknown. However, this could have implications regarding their delousing efficacy when held in cohabitation with Atlantic salmon in sea cages. Similarily, in the current study, inactivity of cunners in the LWT groups was observed throughout the experiment. Additionally, as was discussed in Chapter 3, the lack of delousing performance of cultured juvenile cunner during this experiment could be due to the experimental fish not having developed a natural delousing ability because they lacked a sufficient "learning period" (i.e., the acclimation period with Atlantic salmon smolts in tank environment was insufficient). Furthermore, due to evidence mentioned in Chapter 3, their delousing ability could be parentally

controlled and they did not inherit this from their wild-caught cunner brood stock. The two water temperatures (18ºC and 2ºC) tested in this experiment may have been too warm or too cold, consequently suppressing or even ceasing their delousing performance. According to the description of the cunner breeding program in Newfoundland at the Ocean Sciences Centre, under hatchery conditions, the water temperature for holding wild-caught cunner broodstock in flow-through saltwater is 10-12ºC, egg incubation occurs at 11-12.5ºC, larval rearing at 12-13ºC and juvenile cunner are maintained within between 10-14ºC (Boyce et al., 2018). Water temperature values outside this temperature range (i.e., 18ºC and 2ºC) were chosen for this experiment due to the fact that during the summer months the water temperature in sea cage sites off the coast of Newfoundland can reach as high as 18-20ºC (Kelly et al., 2014). It is unknown whether or not high water temperatures have the ability to negatively impact their delousing behaviour. Additionally, evidence had shown that wild cunner inhabiting the coastal waters off Newfoundland enter a state of torpor and remain inactive at about 5ºC, as determined via field observations (Green & Farwell, 1971). Laboratory studies have shown that adult (>100g) wild-caught cunner captured from coastal Newfoundland waters commenced metabolic depression when water temperature reached below 7ºC and gradually turned torpid with a minimal energy requirement at 3ºC (Kelly et al., 2014). Earlier research suggests that wild-caught cunner can cope with a rapid decrease in temperature (from

14ºC to 0ºC in 2 hours) in the laboratory and are able to withstand temperatures below 0ºC both in the field and under laboratory conditions (Green & Farwell, 1971).

In this experiment, while the acclimation period for both fish species was the same, it may have been too short for the cultured juvenile cunner, which may have resulted in an inability for them to conduct delousing at water temperatures of 18ºC and 2ºC. In order to synchronize the development of *L. salmonis* attached on Atlantic salmon smolts in experimental tanks of all four treatment groups, the water temperature of all experimental tanks remained at 10ºC for approximately 3 weeks post-artificial sea lice parasitism, during which time *L. salmonis* developed to mobile stages (i.e., pre-adult and adult stages), while water temperature adjustments commenced approximately 5-8 days prior to the T0 (pre-addition of cunner). As described in the Methods section, the water temperature was gradually increased or decreased from 11 ± 1ºC to 18ºC (for HWT treatment groups) or 2ºC (for LWT treatment groups), respectively. In previously published research studying cunner physiology, a temperature increase at a rate of 2ºC per day was physiologically tolerable for wild-caught cunner (Kelly et al., 2014), whereas it is unknown whether or not it was physiologically tolerable to the group of cultured juvenile cunners employed in this experiment. Although no acute responses or mortalities occurred in the research population of cultured juvenile cunners, there is a possibility that they may have been stressed in response to the water temperature changes and their

delousing performance could have been suppressed or inhibited with respect to this water temperature adjustment process.

The detection of pathogens in cultured juvenile cunner and Atlantic salmon smolts held at at 2°C and 18°C for the 7 day experimental period was also evaluated. The pathogen screening did not reveal any known pathogens. As described by Boyce et al. (2018), a Canadian cleaner fish health surveillance program was undertaken in the hatchery from which the cultured cunner used in the current study were sourced. This included routine diagnostic sampling (every 45 days) and pre-transfer assessment (approximately 30-45 days prior to the cleaner fish transfer to sea cage sites).

4.6 Conclusion

In the current experiment, neither high or low water temperature (18ºC and 2ºC) nor the presence of cultured juvenile cunner (10% cunner) had a significant effect on the mean sea lice number on Atlantic salmon smolts after 7-days of cohabitation. As such, it was postulated that the group of cultured juvenile cunners used in this experiment did not exhibit delousing behaviour when held in water temperatures of 18ºC (HWT) and 2ºC (LWT) with Atlantic salmon smolts over a 7 day period of cohabitation. The absence of delousing behaviour was also confirmed by the physical examination of the digestive tract of the 30 cultured juvenile cunners which were examined at the end of the

experiment; no sea lice or any other fragments of digested sea lice were observed in their digestive tracts. The results of the pathogen prevalence testing indicated that no pathogens were detected in this group of cultured juvenile cunners and Atlantic salmon smolts. By comparing the test results of fish samples taken before and after cohabitation, the results suggested that cultured juvenile cunners and Atlantic salmon smolts used in this experiment remained healthy in water temperatures of 18°C (HWT) and 2°C (LWT) during 7 days of cohabitation.

Due to the fact that the group of cultured juvenile cunner employed in the current study did not demonstrate delousing activity when cohabitated with Atlantic salmon smolts under laboratory conditions at temperatures of 18ºC and 2ºC over a 7 day of cohabitation, further studies are required.

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CHAPTER 5. Summary

5.1 Summary of research findings

Lepeophtheirus salmonis and *Caligus* spp. (Copepoda: Caligidae), naturally occurring marine ectoparasites that are generically referred to as "sea lice", have been serious and persistent pathogens of concern for Atlantic salmon aquaculture globally (Pike & Wadsworth, 1999; Igboeli et al., 2014). The significant fish health and economic impacts that these parasites, especially *L. salmonis*, have on Atlantic salmon farming industries in the northern hemisphere (e.g., Norway, Scotland and Canada), has necessitated the development of various methods for sea lice control and management. Wild-caught wrasse (e.g., goldsinny, corkwing, cuckoo, rock cook wrasse) and cultured cleaner fish (e.g., ballan wrasse and lumpfish) have been studied and used in Europe as a biological method of sea lice control and mitigation in Atlantic salmon aquaculture, often being used in combination with chemotherapeutants (Treasurer, 2018). Cunner (*Tautogolabrus adspersus*) is the only native species of Labridae found in waters of eastern Canada. Cultured cleaner fish are a more sustainable way to supply cleaner fish for use by the Atlantic salmon aquaculture industry in Atlantic Canada as a wild fishery has not been established (Boyce et al., 2018). Notwithstanding the evidence that some Canadian research groups have verified and documented the delousing ability of wild-caught cunner in laboratory and field trials, limited published information is available with respect to the potential for cultured cunner as a cleaner fish species candidate (DFO,

2014; Costa et al., 2016). This pre-commercial proof-of-concept research was designed to investiage the delousing efficacy of cultured juvenile cunner through an evaluation of the effect of stocking density and water temperature on their delousing performance, including an evaluation of the welfare (e.g., fin condition) and health (e.g., pathogen prevalence) of Atlantic salmon smolt and cultured juvenile cunner held in cohabitation.

The establishment of a sea lice culture system under laboratory conditions (from egg strings to copepodids) and a method to enumerate *L. salmonis* copepodids, demonstrated that the sea lice culture system allowed for the successful development of *L. salmonis* egg strings through to infective copepodids under laboratory conditions. The sea lice culture system designed and employed for this research can be used by future researchers as it was functional, easy to operate, simple and economical to constuct, and suitable for research requiring artificial sea lice parasitism. In order to ensure a consistent and adequate supply of copepodids, several improvements could be made to the sea lice culture system (described in Chapter 2). These include, but are not limited to, applying a saltwater flow-through set-up to afford access to a continuous exchange of saltwater (to optimize water quality). Additionally, a continuous water temperature monitoring system could be employed during sea lice culturing to ensure a constant temperature is maintained (to avoid temperature fluctuations during culturing). Furthermore, modifications could be made to the small hatching units to include an upflowing water

current (i.e., aeration) to ensure sea lice egg strings are continuously suspended in the water column (affording access to an adequate oxygen supply and ensuring better distribution of egg strings within the small hatching units).

The effect of stocking density of cultured juvenile cunner (i.e., 0, 4 and 10% cunner) on their delousing efficacy was evaluated by conducting sea lice counts and comparing the results of three treatment groups over time, along with using fin erosion scores to evaluate the fin condition (e.g., dorsal and caudal fin) of Atlantic salmon smolts as an indicator to verify the potential impacts of the presence of cultured juvenile cunner on the welfare of Atlantic salmon smolt in relation to any inter- or intra- species interactions. The results indicated that there was no significant effect of cultured juvenile cunner stocking density (i.e., 4 and 10%) on the mean sea lice number per Atlantic salmon smolts when held in cohabitation for seven days (p=0.143). However, further investigation into higher densities, while considering fish welfare, could be examined. While there was no evidence of impacts on fish welfare during cohabitation,. if a fin erosion score is adopted as an indicator of fish welfare when Atlantic salmon and cleaner fish are held in cohabitation, it is important to take it into consideration the fact that hatchery sourced fish may have some pre-existing level of fin erosion prior to cohabitation with the cleaner fish species.

The effect of water temperature (i.e., 2 and 18ºC) on the delousing efficacy of

cultured juvenile cunner was evaluated by conducting sea lice counts and comparing the results among four treatment groups over time. While these temperatures represent low and high values that cultured Atlantic salmon and cunner may be exposed to in waters of Newfoundland and Labrador, further experimentation at temperatures within this range (relvent to local water temperature profiles) could be investigated.

The prevalence of several commercially significant viral and bacterial pathogens was determined by submitting fish samples of Atlantic salmon smolts and cultured juvenile cunner from the research population to accredited provincial fish health laboratories. The results indicated that there was no significant effect of treatment (p=0.093) on the mean sea lice number per Atlantic salmon smolt when held at 18ºC (HWT) and 2ºC (LWT) at densities of 0 and 10% cunner for seven days. There were no detections of any pathogens and no clinical signs of disease were observed, which implied no evidence of the prevalence of commercially significant pathogens in either species during the cohabitation.

In summary, the results indicated that the cultured juvenile cunner used in this research did not exhibit delousing behaviour when held in cohabitation with Atlantic salmon smolts under laboratory conditions for a period of seven days. Additionally, the presence of cultured juvenile cunner did not pose any threat to the welfare of Atlantic salmon smolts and there was no evidence of impacts to fish welfare of Atlantic salmon,

nor was there any evidence of the prevalence of disease during seven days of cohabitation.

5.2 Future research

Due to the fact that the group of cultured juvenile cunner employed in the current study did not demonstrate delousing activity at different stocking densities (i.e., 4 and 10%) or different water temperatures (2 and 18ºC), further studies are required to verify their delousing efficacy at different stocking densities and temperatures. An investigation into the requirement for a period of acclimation prior to the commencement of such studies is required, as previous research suggests that the sea lice grazing activity of cultured cleaner fish may be a learned behaviour and there may be a genetic component associated with delousing which would allow for the study of cultured cunner with known delousing abilities (Powell et al., 2018). Given the fact that cultured juvenile cunners applied in this experiment were off-feed for only two days prior to cohabitation with sea lice infected Atlantic salmon smolts, it could be suggested that a longer period off-feed could induce hunger (for cunner) and improve their feeding reponse toward sea lice. Furthermore, given the fact that the sea lice numbers were low in Experiment 2 (Chapter 4), this may also have influenced cunner feeding behaviour. Analysis of the stomach contents of cultured juvenile cunner could provide evidence and confirmation of sea lice grazing activity (Imsland et al., 2014; 2015). The ability to PIT tag individual

study fish to more precisely track repeated sea lice counts of individual Atlantic salmon smolts over time would enhance the statistical power of future research studies. The establishment of a breeding program is recommended to facilitate the selection of progeny which are derived from confirmed lice-eating families prior to deployment in sea cages for large-scale application. Finally, a disease surveillance program is recommended for cultured cleaner fish that are intended to be cohabitated with Atlantic salmon in sea cages, as is currently in place in Newfoundland and Labrador (DFFA, n.d.).

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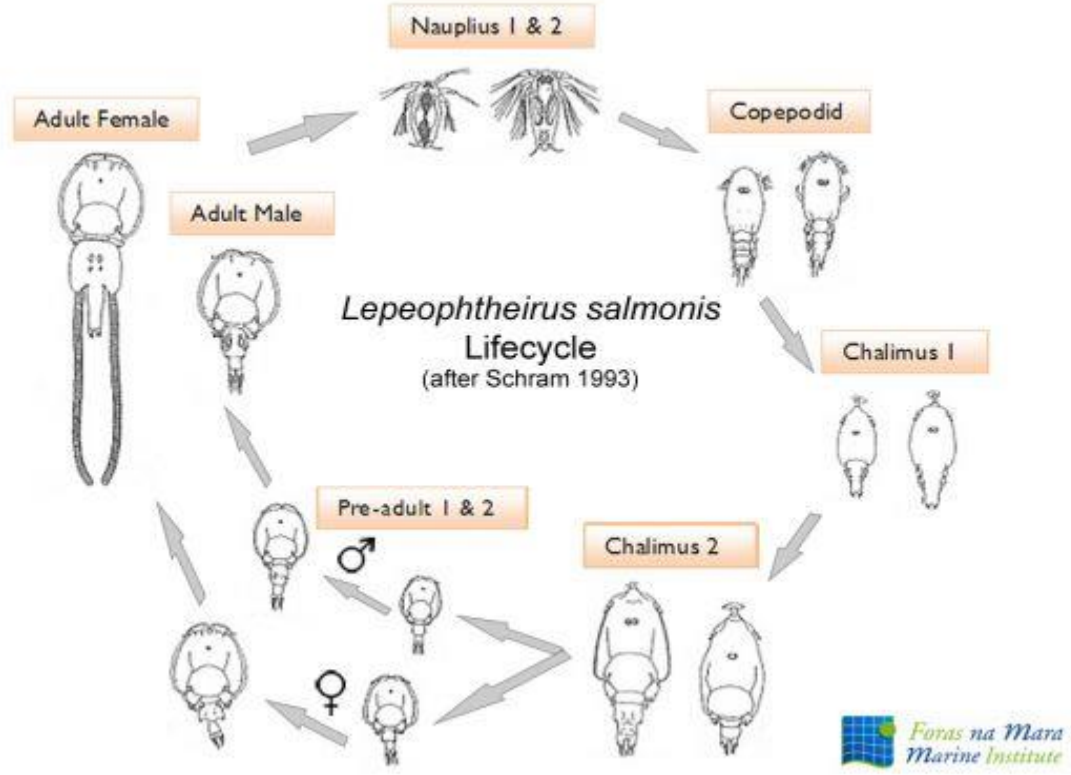
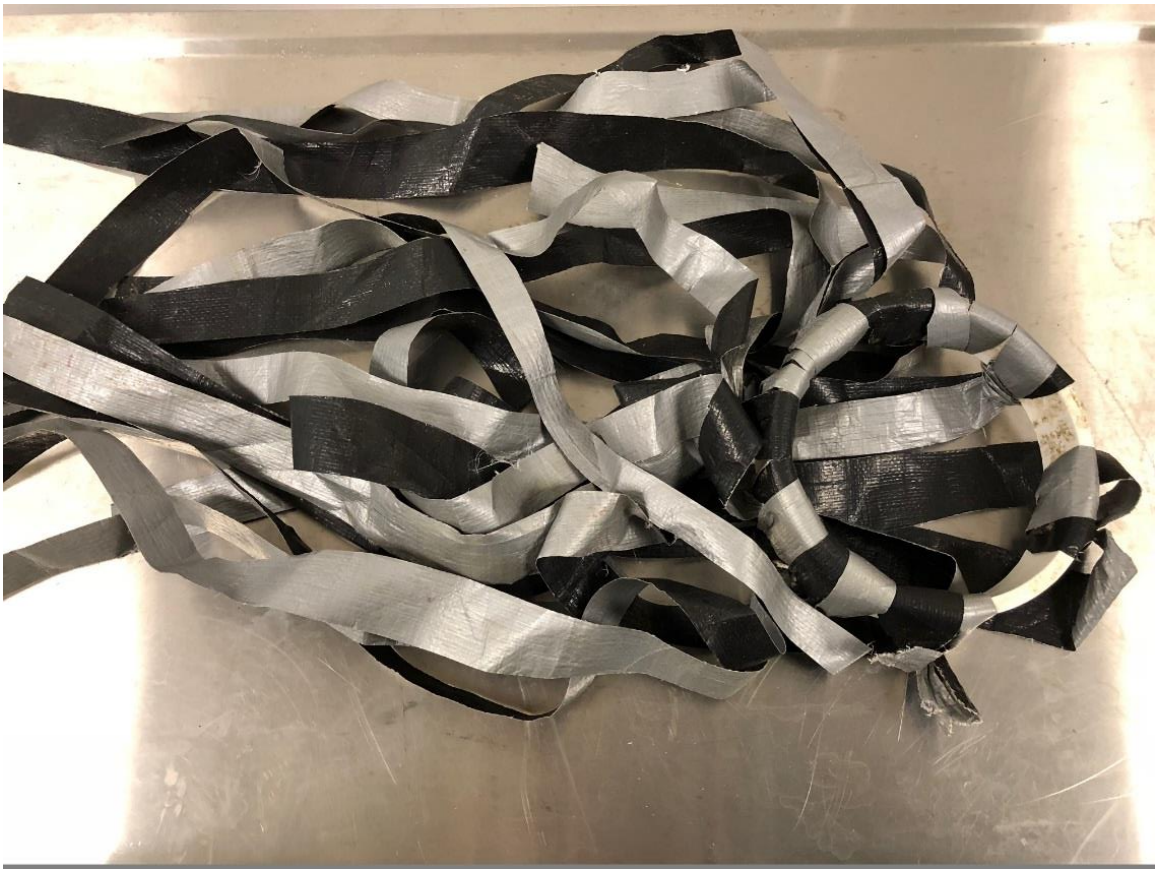
APPENDICES



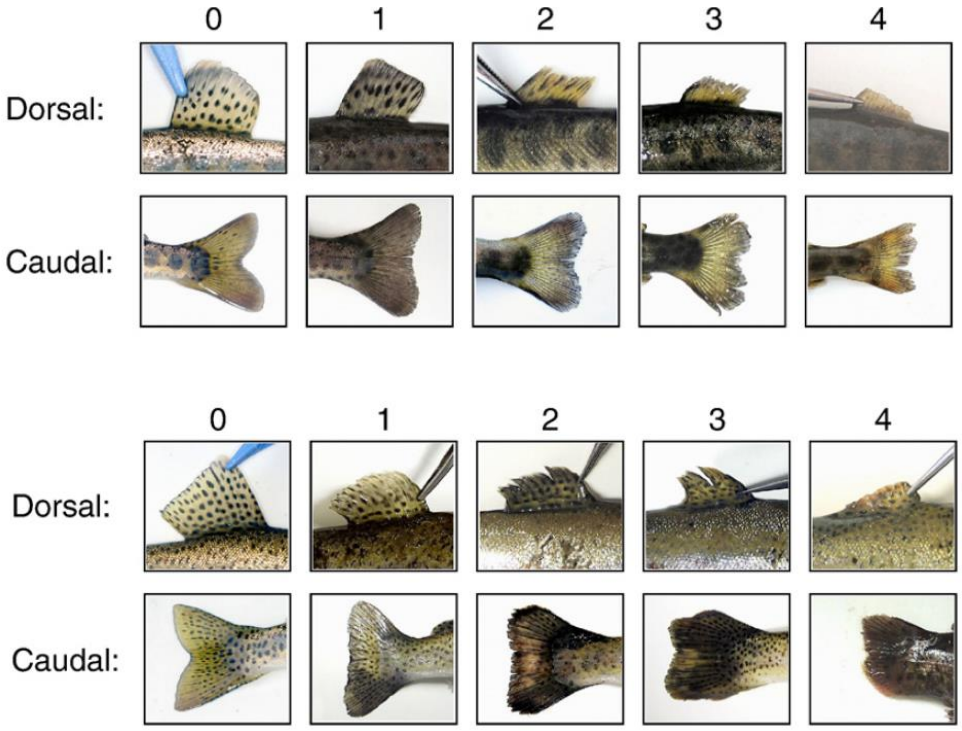
Appendix A. Updated life cycle of *Lepeophtheirus salmonis* with eight developmental stages (Marine Institute, Ireland, 2020).

Appendix B. Chemotherapeutants employed for sea lice control in major Atlantic salmon farming countries.

ACTIVE COMPOUND	CHEMICAL CLASS	BRAND/TRADE NAME	RECOMMENDED DOSAGE OR CONCENTRATION	TREATMENT TIME	MODE OF ACTION	TARGET STAGE OF SEA LICE	REFERENCE(S)
BATH TREATMENTS							
Dichlorvos	Organophosphate	Nuvan® Aquagard®	1 mg/L	Up to 60 mins	Interferes with nerve transmission by blocking acetylcholinesterase at synapse	Pre-adult and Adult only	Costello, 1993; Burridge et al., 2010
Azamethiphos	Organophosphate	Salmosan®	0.1 mg/L	Up to 60 mins	Interferes with nerve transmission by blocking acetylcholinesterase at synapse	Pre-adult and adult only	O'Halloran & Hogans, 1996; Roth, 2000; Grant, 2002; Burridge et al., 2010
Cypermethrin	Pyrethroid	BetaMax® Exics®	15 µg/L (BetaMax®) 5 µg/L (Exics®)	30-60 mins	Interferes with nerve transmission by blocking sodium channel in nerve cell	Chaliums I & II, pre-adult and adult	Roth, 2000; Burridge et al., 2010
Deltamethrin	Pyrethroid	AlphaMax®	2-3 µg/L	40 mins	Interferes with nerve transmission by blocking sodium channel in nerve cell	Chaliums I & II, pre-adult and adult	Roth, 2000; Burridge et al., 2010
Hydrogen Peroxide	Oxidizer	Paramove® Salartect®	0.5-1.5 g/L	20 mins shorter time at higher temperature	Gas embolism; efficacy is temperature dependent (shorter time at higher temperature)	Pre-adult and adult only	Burridge et al., 2010; Igboeli et al., 2014; Overton et al., 2018
IN-FEED TREATMENTS							
Diflubenzuron	Benzoylurea	Lepsidon®	3 mg/kg/day	14 days	Chitin synthesis inhibitor preventing moulting and growth	Chalimus & pre-adult	Roth, 2000; Grant, 2002; Burridge et al., 2010
Teflubenzuron	Benzoylurea	Calicide®	10 mg/kg/day	7 days	Chitin synthesis inhibitor preventing moulting and growth	Chalimus & pre-adult	Grant, 2002; Burridge et al., 2010
Lufenuron	Benzoylurea	Imvixa®	5mg/kg/day	7 days	Chitin synthesis inhibitor preventing moulting and growth	Chalimus & pre-adult	Poley et al., 2018
Emamectin Benzoate	Avermectin	Slice®	0.05 mg/kg/day	7 days	Interferes with nerve transmission by blocking sodium channel in nerve cell causing paralysis and death	All developmental stages	Roth, 2000; Treasurer et al., 2000; Grant, 2002; Burridge et al., 2010



Appendix C. Photo of artificial "hide" deployed in holding tanks for cultured juvenile cunner.



Appendix D. The 5-point fin erosion index with photographic keys used for evaluating fin erosion scores of Atlantic salmon smolts (modified from Hoyle et al., 2017). Fin erosion index can be applied to both sides of dorsal and caudal fins, where, 0=no erosion, 1=slightly eroded, 2=moderately eroded, 3=half fin eroded, 4=severely eroded.