Pacific Oyster (*Crassostrea gigas*) and Atlantic Salmon (*Salmo salar*) Integrated Multi-Trophic Aquaculture in British Columbia: Investigation of Bivalve Growth and Natural Sea Lice Mitigation

by

Allison Byrne
BSc, Mount Allison University, 2012

A Thesis Submitted in Partial Fulfillment
of the Requirements for the Degree of

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Supervisory Committee

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Abstract

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The close proximity of net-pen salmon farms and wild Pacific salmon stocks in British Columbia (BC) is an incentive for precautionary management of the environmentally and economically damaging parasites known as sea lice. Bivalves cultured as part of an integrated multi-trophic aquaculture (IMTA) system may contribute natural, preventative louse control through the ingestion of planktonic sea lice larvae. A field trial was conducted to test sea lice mitigation by bivalves at a commercial Atlantic salmon (*Salmo salar*) farm in BC using Pacific oysters (*Crassostrea gigas*). Oysters were cultured in trays around one end of the farm and at a reference site approximately 150 m away from August 2013 until August 2014.

Parasitic and planktonic sea lice (*Lepeophtheirus salmonis* and *Caligus clemensi*) were monitored before and during oyster deployment, beginning in December 2012. Parasite abundance peaked in January 2013 (6.5 lice·fish^{-1}, >85% *C. clemensi*), and the following year in February 2014 (3.3 lice·fish^{-1}, >80% *L. salmonis*). Larval density within cages peaked in January, both in 2013 (1.28 larvae·m^{-3}) and 2014 (0.96 larvae·m^{-3}). Parasite abundance was significantly correlated with both surface salinity (r^2= 0.28, p=0.04) and sea lice larval density (r^2= 0.65, p=0.01). Observed densities were significantly lower (t=3.41, p=0.009) than those calculated for the site based on water

temperature and salinity, the number of adult female lice present, and the approximate number of fish.

Sea lice mitigation by oysters was assessed by comparing monthly sea lice larval densities inside bivalve and non-bivalve fish cages, and by analyzing preserved oyster digestive tracts from January 2014 (when larval densities were highest) for presence of *L. salmonis* DNA using PCR. Using these methods, no significant evidence of sea lice mitigation was detected. Oyster growth was monitored by measuring whole wet weight, soft tissue wet, dry, and ash-free dry weight, and shell length, width, and height approximately every four months. Oysters were sampled equally across different sides of the farm and at the reference site (~150 m away from the farm) at three depths: 1, 3, and 6 m. All seven measurements increased significantly over time. Effects of side and depth varied by growth parameter; in general, oysters at 1 and 3 m were significantly larger than those at 6 m, and oysters cultured at the reference site were either significantly smaller or the same size as those cultured around the farm. Oysters from select sides were consistently, significantly larger than those from other sides and from the reference site.

Overall, the findings suggest that sea lice larvae quickly dispersed away from the farm after hatching and were not significantly impacted by bivalve presence around the fish cages. Bivalves grew significantly larger over time and size was significantly impacted by both depth and side of the fish cage. While no evidence of larval sea lice reduction/ingestion by cultured bivalves was detected, this study provides information on all sea lice stages present throughout an Atlantic salmon production cycle, as well as the first detailed growth analysis of Pacific oysters cultured alongside farmed Atlantic salmon in BC.

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Abbreviations

General

AFDW – Ash-free dry weight
BC – British Columbia
BP – Base pairs
DFO – Department of Fisheries and Oceans Canada
DNA – Deoxyribonucleic acid
DW – Dry weight
IMTA – Integrated multi-trophic aquaculture
IPM – Integrated pest management
MS-222 – Tricaine methane sulphonate
PBS – Pacific Biological Station
PCR – Polymerase chain reaction
TP – Tissue positive (oyster spiked with *L. salmonis* copepodids)
TVS – Total volatile solids
WW – Wet weight

Statistics

ANOVA – Analysis of variance
BF – Blocking factor
DF – Degrees of freedom
GLM – General linear model
JMP® – Statistical software by SAS®
Ln – Natural logarithm (data transformation)
SD – Standard deviation
SE – Standard error of the mean
SNK – Student-Newman-Keuls (multiple comparisons test)

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Chapter 1: Introduction

The aim of this chapter is to provide background on three overarching themes of this Master's thesis: 1) salmon aquaculture, 2) sea lice, and 3) integrated multi-trophic aquaculture (IMTA). Discussions of salmon aquaculture and sea lice will emphasize information relevant to British Columbia (BC), where the project was conducted. IMTA will first be introduced broadly, followed by a more in-depth examination of specific research on IMTA filter-feeding bivalves. The Chapter will then conclude by presenting the project's four central research questions.

Salmon Aquaculture

Aquaculture is a diverse global industry growing both in terms of production and importance as a food-producing sector; human consumption of seafood products is higher than ever yet the majority of wild capture fisheries are either stagnant or declining (FAO, 2014). As a whole, the aquaculture industry is dominated by species that rely fully or partially on nutrients from the surrounding environment for growth such as macroalgae, shellfish, and herbivorous or omnivorous pond fish (Bostock et al., 2010). Salmon and other marine finfish represent only 7% of all aquaculture species for both weight and value (FAO, 2010), but have a unique importance due to the resource inputs required (*i.e.* feed and fossil energy), relatively high-value end products, and public controversy surrounding their production (Naylor et al., 2009).

Atlantic salmon (*Salmo salar*) is the leading intensively-farmed marine fish (Bostock et al., 2010) owing to its success under high stocking density conditions, fast-

growing nature, and established international markets (Saksida et al., 2011b). Salmon farms provide a reliable source of fresh, nutritious fish year-round unlike the wild salmon fishery where catches coincide with annual spawning periods. Norway, Chile, Scotland, and Canada are the largest producers of farmed Atlantic salmon (FAO, 2010) and generally use floating cages in coastal waters – the most cost-effective means of culturing salmon or other marine finfish at present (FAO, 2007). These sea cages (also called net pens) are continually replenished with new, oxygenated seawater though by consequence allow the release of nutrients, chemicals, and pathogens (if present) into the marine environment (Burridge et al., 2010; FAO, 2007), a characteristic that will be important in later sections on sea lice and IMTA.

Salmon Aquaculture in British Columbia

The emergence of large-scale commercial salmon farms has altered, for better or worse, many coastal landscapes and communities in BC. Early attempts at farming Pacific salmon (*Oncorhynchus* spp.) in the 1970's were unsuccessful overall, due largely to farmer and/or regulator inexperience on matters such as disease control, fish husbandry, and strategic farm siting (DFO, 2010). Beginning in 1984 the province was permitted to import Atlantic salmon eggs, a species already being farmed successfully in Norway and Atlantic Canada (DFO, 2010). There are now 123 approved finfish farm tenures in BC (Brewer-Dalton et al., 2015), the vast majority of which are licensed for Atlantic salmon (other finfish including sablefish, Chinook salmon, and rainbow trout are also being farmed). Approximately 70—90 of the licenced finfish tenures are stocked with fish at a given time (Saksida et al., 2011b). Most tenures are located on the east

coast of Vancouver Island from Campbell River north to Port Hardy and throughout inlets along the north-west coast of Vancouver Island.

Despite being introduced only three decades ago, farmed Atlantic salmon has become the province's largest agricultural export and boasts the highest harvest value of any species (Saksida et al., 2011b). Atlantic salmon farms in BC are licenced for 100—5258 tonnes combined peak biomass (average site ~2500 tonnes) (DFO, 2015a). In 2013, salmon aquaculture revenue in BC was $476 million CAD, representing 94% and 51% of the total provincial and national aquaculture revenue, respectively (Statistics Canada, 2013). Salmon farming employs upwards of 6000 British Columbians while industry expenditures contribute hundreds of millions of dollars into local businesses each year (*e.g.* equipment suppliers, shipping and marketing companies) (DFO, 2010). Much of this economic gain befalls small coastal communities that have restricted opportunities for commercial development.

The decision to import and farm a non-native fish in BC waters raised complicated ethical questions and environmental concerns that persist today. British Columbia is unique in that Atlantic salmon farms and several native Pacific salmon and trout species (*Oncorhynchus* spp.) co-exist. Pacific salmonids are also valuable to the provincial economy through the commercial and sport-fishing industries. However, unlike farm Atlantic salmon, these fish provide a multitude of cultural, regulating, and supporting ecosystem services unrelated to direct financial payoff (Gende et al., 2002; Schindler et al., 2003). Most of the public's criticisms of salmon aquaculture in BC relate to farm-wild salmon interactions and specifically the notion that farm salmon may harm wild Pacific salmon stocks and/or their habitat (Cohen Commission, 2011).

Environmental Impacts of Salmon Aquaculture Wastes

Organic wastes from salmon farms, namely excess fish feed and faeces, may accumulate on the ocean floor and alter natural processes and the habitat of native organisms. The magnitude of any adverse environmental effect(s) is site-specific based on parameters such as water depth (Kutti et al., 2007), current speed, salinity, and rainfall (Brooks and Mahnken, 2003). Salmon feed is efficiently converted into somatic tissue with modern feed formulations which are 87—88% digestible (Brooks and Mahnken, 2003). An estimated 3% of salmon feed goes uneaten (Cromey et al., 2002; Reid et al., 2009) which constitutes approximately 12—17% of the total solid waste from food – the majority of solid waste coming from faeces (Reid et al., 2010, 2009). Solid organic wastes generally settle locally, either directly beneath or in close proximity to the cages (Brooks, 2001) and, similarly, suspended organics from finfish farms have shown periodic enhancement only within or directly beside fish cages (Brager et al., 2015). In addition to organic wastes, fish farms produce inorganic waste nitrogen and phosphorus compounds. An estimated 35 kg nitrogen is released (through respiration, urine, and faeces) per tonne of fish produced (Wang et al., 2013). The largest fraction of this is excreted as dissolved inorganic nitrogen (DIN, 39—45%) (Wang et al., 2013, 2012). Dissolved inorganic nitrogen from salmon farms is essentially undetectable at 30 m downcurrent from the net pens and has no measurable effect on phytoplankton (Brooks and Mahnken, 2003). Despite rapid dilution, perhaps making nutrient enrichment inappreciable, waste from one salmon farm producing >100 tonnes is, from an ecological

perspective, the equivalent of hundreds of persons' worth of municipal sewage in terms of its potential to pollute and cause eutrophication in coastal waters (Folke et al., 1994).

Sedimentation rates of total volatile solids (TVS) around salmon farms has remained in the range of 15—100 g TVS $m^{-2} \cdot d^{-1}$ from the 1980's through 2000's (Brooks and Mahnken, 2003). Farms in BC must monitor TVS at peak biomass both on-site and at reference stations (30 m and 125 m away) if the site is above a soft sediment substrate (DFO, 2013). These reports are made available to the public (DFO, 2015b). Kutti et al. (2007) reported that the deposited organic nutrients at a farm located at a deep site (230 m) in Norway did not exceed capacity for natural remediation by the benthic community. However, high amounts of salmon faeces and large numbers of sea urchins and polychaetes were observed in the benthos adjacent to the farm versus 1.5 km away (Kutti et al., 2007). Organic wastes from farm salmon are known to build up and provide nutrients for infauna, increasing secondary production in the benthos (Kutti et al., 2008). Problems can arise in areas subjected to prolonged organic enrichment where the normal benthic community may, over time, transition into a community of low biodiversity, *i.e.* few opportunistic species in high abundance (Pearson and Rosenberg, 1978). What constitutes a "normal" community is predictable for a particular habitat but varies among habitats, as do the opportunistic species (Pearson and Rosenberg, 1978). Azoic sediment arises when the deposition of organic matter surpasses the decomposition rate by even the opportunistic benthic fauna and microorganisms. Based on a study of salmon farms in Maine, sedimentation rates between 200 and 400 mmol $C \cdot m^{-2} \cdot d^{-1}$ (2.4—4.8 g $C \cdot m^{-2} \cdot d^{-1}$) serve as the threshold for observing azoic sediments (Findlay and Watling, 1997).

The organic assimilative capacity of the benthos is reached when the rate of oxygen consumption equals that of the oxygen infusion (Brooks and Mahnken, 2003). Oxygen is used for organic decomposition both on the sediment surface during aerobic respiration and within the sediment where the major oxygen-consuming process is the oxidation of hydrogen sulfide into sulfate (Brooks, 2001). Hydrogen sulfide is produced when organic matter is anaerobically decomposed by sulfate-reducing bacteria (Findlay and Watling, 1997). Sediment free sulfides are significantly correlated with farmed salmon biomass, feeding rates, TVS deposition, and biological endpoints *e.g.* taxa diversity (Brooks, 2001). Opportunistic annelids (*Schistomeringos* sp., *Capitella capitata*, and *Sigambra tentaculata*) and crustaceans (*Nebalia pugettensis, Aoroides* sp., and *Pseudotanais oculata*) are found in areas of high sulfide concentrations, $\leq 5{,}000$ μM (Brooks, 2001). Sulfide-sensitive animals such as small mollusks are absent in the immediate vicinity of salmon farms in BC (Brooks and Mahnken, 2003; Brooks et al., 2003). Current finfish aquaculture licenses in BC require farms above soft sediment substrates to monitor free sulfides during peak biomass and prior to re-stocking, both at the farm and reference sites (30 m and 125 m away, as with TVS) (DFO, 2013). Fallowing between harvest and re-stocking allows chemical (sulfide and redox) and biological (infaunal community) remediation, and typically takes several months (Brooks et al., 2003).

Sea Lice

Controlling sea lice is another major area of criticism that remain a challenge for the salmon aquaculture industry in BC as well as globally. The term “sea lice”

encompasses hundreds of species of parasitic caligid copepods that are naturally present in marine and brackish ecosystems on a wide variety of fish hosts (Boxshall and Defaye, 1993). Host preference is dependent on the species of louse; for example, *Caligus elongatus* is found on at least 34 families of fishes (Parker, 1969), whereas *Lepeophtheirus salmonis* is specific to the family Salmonidae (though its presence on non-salmonids in BC has been reported, Jones and Prosperi-Porta (2011); Jones et al. (2006)) (Kabata, 1973). Sea lice feed on fish skin, mucus, and in some cases blood, which can result in scale loss and epidermal lesions (Wagner et al., 2008). Sufficient damage to the fish's critical skin barrier can lead to physiological stress, most notably compromised osmoregulation, and a probable increased risk of contracting secondary infections (Finstad et al., 2000; Grimnes and Jakobsen, 1996; Wootten et al., 1982). Two species comprise virtually all sea lice present on farmed salmon in BC: *Lepeophtheirus salmonis* and *Caligus clemensi* (Figure 1). The former is more damaging than the smaller *C. clemensi* which mostly surface grazes.

Sea lice develop from eggs into free-swimming, non-feeding larvae that must infect a host before their energy reserves are exhausted. Once attached, a series of parasitic stages ultimately generates reproductive adults. Mature males preferentially seek out pre-adult or virgin adult females (likely aided by chemical signals given off by the female) and exhibit mate guarding until copulation (Ritchie et al., 1996). From one mating event a female may produce more than 10 sets of egg strings which are extruded in pairs (Heuch et al., 2000). Fecundity (the number of eggs per string) is highest in the winter months for *L. salmonis* (Ritchie et al., 1993; Tully, 1989) and is generally in the range of 200–400 eggs per string (Heuch et al., 2000; Wootten et al., 1982). Further

differences in reproductive output between female lice on farm versus wild hosts, or on different species of salmonids, have also been reported (Johnson, 1993; Tully and Whelan, 1993). Hatching and development through the life stages is very much temperature-dependent (Heuch et al., 2000; Johnson and Albright, 1991) and requires high salinity, ideally 30 PSU or higher for active copepodids that are capable of successful host infection (Bricknell et al., 2006; Johnson and Albright, 1991). The total generation time of *L. salmonis* is an estimated 7.5–8 weeks at 10˚C (Johnson and Albright, 1991).

Distribution of Sea Lice Larvae

Together, two nauplius stages and one copepodid stage comprise the larval phase of the sea louse life cycle (Figure 2). Salmon farms experiencing lice infestation can add millions of sea lice nauplii to the surrounding environment each day (Orr, 2007; Tully and Whelan, 1993). The first nauplius moults quickly – within hours to days – into the second nauplius which is anatomically similar but with a slightly longer, more tapered body. In 1.5–7 days the second nauplius will moult into a copepodid (Johnson and Albright, 1991), with nauplii perhaps seeking warmer waters to expedite their transition into the infective phase (Norði et al., 2015). Copepodids may persist for extended periods of time unattached to a host and thus still non-feeding (10 days in the laboratory, personal observation). Attachment of copepodids onto a fish host marks the transition into the parasitic phase consisting of chalimus, pre-adult, and adult stages.

Lepeophtheirus salmonis copepodids are able to target salmonid hosts using a combination of sensory organs including eye spots (associated with light attraction),

mechanoreceptors (to detect water movement or vibrations), and antennules containing chemoreceptors (to verify host species) (Bron et al., 1993). In addition to having a specialized sensory system, copepodids also display behaviour amenable to host-finding under experimental conditions, namely a vertical migration to surface waters during the daytime and falling to greater depths at night (Heuch et al., 1995). This may increase parasite encounter rates with farm salmon which feed near the surface during the day (Heuch et al., 1995).

The ability of larvae to move between farmed and wild fish populations – a huge issue affecting the Atlantic salmon farming industry in BC – is largely dictated by local hydrology and is eventually limited by the longevity of these endogenously-feeding stages (Tully and Nolan, 2002). For successful attachment, copepodids benefit from moderate water currents that optimize host-parasite interaction; high (36.7 cm s^{-1}) and low (5.1 cm s^{-1}) currents and fast-swimming fish hindered louse infection success in the laboratory (Samsing et al., 2015). Most sea lice dispersion models limit travel distances to 30 km or less (Krkošek et al., 2005). Modeling larval dispersion is challenging as one must incorporate biotic and abiotic factors in a complex marine environment (*e.g.* Kristoffersen et al., 2014; Murray and Gillibrand, 2006; Stucchi et al., 2011). Moreover, observational data with which to validate models may be limited or absent as larvae are rapidly diluted and generally difficult to detect away from farms (Costelloe et al., 1996; Penston et al., 2004).

A study on the Broughton Archipelago region of BC combined egg production from active farms with water circulation, temperature, and salinity to estimate copepodid abundance (Stucchi et al., 2011). Predicted levels of the infective larvae were usually less

than 0.1 m^{-3} and lower than field observations in the area which were, however, limited in terms of sample size and geographic extent (Stucchi et al., 2011). Areas with very low predicted levels of copepodids coincided with both no copepodids found in plankton samples as well as an absence of lice on wild juvenile salmon (Stucchi et al., 2011). It has been noted that the hydrodynamic regime in the Broughton Archipelago "is among the most complex in the world for aquaculture use" (Foreman et al., 2015), driving home the difficulty of this type of analysis. Further understanding of sea lice dispersal in and around farms could have direct impacts on aquaculture policies that help lessen lice-related risks posed by farms to wild fish stocks, for example strategic siting of new salmon farm tenures or creation of sea lice management zones (Foreman et al., 2015). As well, model predictions may increase the efficiency of sea lice mitigation efforts (*e.g.* treatment, fallowing) in a region and help focus resources to high-risk areas.

Sea Lice on Wild Juvenile Salmon

How the host immune system reacts to *L. salmonis* infection varies among salmonid species (Fast et al., 2002; Johnson and Albright, 1992; Jones et al., 2007). Identified pathways of resistance or susceptibility, whether species-specific or shared among multiple species, relate to the host's innate immune response at the louse attachment site (see Braden et al. 2015). Importantly, Atlantic salmon exhibit a weak immune response to *L. salmonis* when compared to the more resistant pink or coho salmon (Fast et al., 2002; Johnson and Albright, 1992; Jones et al., 2007). Net pen Atlantic salmon farms therefore provide high densities of susceptible hosts – favourable conditions for the parasite – creating an opportunity for epizootics

uncommon in nature (Costello, 2009a) (though natural epizootics have been observed, *e.g.* Johnson et al., 1996). Salmon aquaculture sea sites in BC begin production at an empty site (typically fallowed for several months, Brooks, 2009) with fish from land-based freshwater hatcheries, free of any sea lice and other marine parasites. Sea lice therefore enter the farm from wild and/or farm fish in the surrounding environment. Understanding the dynamics of parasite transfer into the farm and subsequent spill-back to wild fish, and mitigating these using chemical and non-chemical means, is important for the health of both farm and wild fish populations.

It has been argued that sea lice from Atlantic salmon farms in BC are increasing infections on wild juvenile salmon leading to their mortality beyond natural levels (Krkošek et al., 2007, 2006), in particular juvenile pink salmon which migrate from rivers to the ocean immediately after hatching (Krkošek, 2009; Morton et al., 2005). These young fish are considered especially vulnerable to sea lice due to their small size (0.2 g) during the transition to salt water (Brauner et al., 2012) and coinciding underdeveloped immune (Finstad et al., 2000; Jones et al., 2008; Sutherland et al., 2011), ionoregulatory (Brauner et al., 2012), and osmoregulatory (Sackville et al., 2011) systems that are important for louse resistance. While less able to shed sea lice, damages incurred from louse attachment and feeding are also proportionately greater for smaller fish (Brauner et al., 2012). Thus, until pink salmon reach 0.5–0.7 g and these systems have developed (Brauner et al., 2012; Jones et al., 2008; Sackville et al., 2011) there exists a window of susceptibility to damaging louse infections (Jones et al., 2008). Quantifying the extent to which sea lice from farm

salmon impact wild salmonids is challenging as these fish naturally experience greater than 95% mortality, *i.e.* the probability that a salmon smolt entering the ocean returns to spawn is approximately 5% (Jones et al., 2015). Regardless, precautionary measures to minimize potential impacts are a central driver of current sea lice management policies in BC (Saksida et al., 2011b) with evidence of success over the last decade (Peacock et al., 2013).

Economic Costs of Sea Lice

Sea lice reduce the profitability of farm salmon in a number of direct and indirect ways. Globally it is estimated that the combined financial losses from sea lice infections total more than $100 million USD each year (Johnson et al., 2004). The largest direct costs surround the purchase and administration of chemical sea lice treatments (Costello, 2009b). Although costly, farms would lose an estimated 3–4 times more money per kilogram of fish by leaving the site untreated (Mustafa et al., 2001). Damages caused by parasite attachment and feeding can also directly lower fish value, forcing a percentage of the harvest to be downgraded (Mustafa et al., 2001). Indirectly, sufficient parasite intensity significantly decreases fish growth and food conversion efficiency (FCE) (Johnson et al., 2004). Feed is the largest operating cost at finfish farms (Naylor et al., 2009), so even a small percentage increase in feed (to compensate for reduced growth and FCE) is a substantial loss. Fish mortality was the least significant contributor to reduced earnings as ranked by Costello (2009b) since most sea lice infections are non-lethal, especially in BC where heavy *L. salmonis* infestations are rare (Saksida et al., 2007a). Economic losses from negative public perceptions of sea lice on farm salmon,

and the chemical treatments thereof, are also notable though difficult to quantify (Costello, 2009b).

Sea Lice Management

As mentioned, farm Atlantic salmon are introduced to the ocean free of sea lice. The onset of sea lice infections can be delayed using fish husbandry techniques such as stocking only a single year-class of fish and allowing a site to fallow prior to re-stocking (to eliminate self-infestation by lice from the previous cycle, Brooks, 2009). Routine monitoring (at least monthly in BC) is an important aspect of any pest management plan. In BC a threshold level of three motile (pre-adult and adult) *L. salmonis* per fish, on average, is reached during the juvenile pink salmon migration from March until July, "action" is required – *i.e.* farms must either be treated or harvested (Saksida et al., 2011b). Efficacy of both preventative (husbandry-related) and reactive (treatment or harvest) sea lice mitigation strategies may be enhanced if multiple farms in a management area synchronize their execution (DFO, 2014). Management areas in BC are called fish health zones which generally follow major watersheds (Saksida et al., 2011b).

Chemical treatments for sea lice fall under five classes of active ingredients: avermectins, pyrethroids, organophosphates, chitin synthase inhibitors, and hydrogen peroxide. Their usage and efficacy varies between and within countries (Burridge et al., 2010). The only approved treatment in BC until 2014 was an avermectin in-feed chemotherapeutant formulation called SLICE® (active ingredient: emamectin benzoate) (Burridge and van Geest, 2014). It remains highly effective almost everywhere in the

province, and for the time being infestations of *L. salmonis* are not recognized as a major health concern (Saksida et al., 2007a). At dosages used by industry, SLICE® is not lethal to any non-target organisms tested to date (Burridge et al., 2010). A large gap in this literature is that no non-target species native to the Pacific have been tested for effects of emamectin.

Using multiple treatments in rotation as part of an integrated pest management (IPM) plan may delay or prevent pathogen resistance. A question arises, though, of whether chemically treating fish displaying no signs of clinical disease is in fact ethical (Saksida et al., 2011b) especially given the inflexible arbitrary louse threshold (Saksida et al., 2015) and evidence that SLICE® has lost efficacy elsewhere in the world (*e.g.* Bravo et al., 2008; Lees et al., 2008). Overall, *L. salmonis* in BC do not appear resistant to SLICE® however bioassays have demonstrated that significant differences in efficacy of the drug do exist between and within farms (Saksida et al., 2013). Researchers therefore stress the importance of continued monitoring and use of alternative treatments that have a different mechanism of action (Saksida et al., 2013). Hydrogen peroxide (in the formulation Paramove 50™) has recently been approved and administered in certain parts of BC (Morrison, 2014).

Integrated Multi-Trophic Aquaculture

This final section will introduce integrated multi-trophic aquaculture (IMTA) which addresses many of the negative issues faced by the expanding finfish aquaculture industry, though it also creates new research challenges in the natural and social sciences (Chopin et al., 2013). IMTA is the farming of aquaculture species from different trophic

levels whereby waste nutrients (uneaten feed and wastes) from one species may be recaptured into energy for other species, taking advantage of synergistic relationships between trophic levels (Chopin et al., 2012). A hypothetical IMTA system would include a fed component such as finfish, with macroalgae to recycle dissolved inorganics, filter feeders to uptake small organic particulates, and deposit feeders to recycle larger organic particulates. A fundamental view of IMTA is that a successful aquaculture system should produce by-products, not wastes, which act as positive contributors to their surrounding ecosystems and to the economy (Folke and Kautsky, 1992). Extractive, low trophic-level species use these by-products (in a variety of ways) together with resources from the environment for growth. In this way IMTA farms produce multiple crops of seafood, theoretically increasing overall economic and ecological efficiency. IMTA is not a new concept *per se* – the commercial mimicry of natural ecosystems has been practiced in Asia for centuries (*e.g.* China, Chan (1993)) – though it stands in contrast to monoculture crops that are the norm in Canadian aquaculture (Chopin, 2015) and in Western farming in general.

Economic and Social Justification for IMTA

Each step up the food chain increases feed and fossil energy inputs, making salmon and other high trophic-level species both economically and environmentally costly (Neori and Nobre, 2012). Economic analysis has shown realistic potential for IMTA practices to increase farm profit margins and lower risk if implemented at finfish aquaculture sites (Ridler et al., 2007). Increased profit margins hypothetically arise from harvesting multiple crops in a staggered fashion, rather than simply at the end of one

species' grow-out cycle. Lower risk primarily refers to the buffer created by cultivating several crops at once, for if one fails or performs poorly in a given year (in price and/or production), other sources of income remain (Chopin et al., 2008). Fortunately, much of the upfront high-cost infrastructure and equipment would already exist at a salmon (or other monoculture) farm and therefore the cost of additional extractive species is likely not additive (Chopin et al., 2008). IMTA could further increase the economic sustainability of coastal communities through job creation (projected in the hundreds for New Brunswick alone) and/or diversification, since the design, construction, and operation of IMTA sites would require a variety of skillsets (Barrington et al., 2009).

In general, focus group participants recognize improvements of IMTA over current aquaculture practices and would welcome it into the marketplace (Barrington et al., 2010; Ridler et al., 2007). A lack of education on the IMTA concept is a significant barrier, however, for its acceptance and implementation (Shuve et al., 2009). As well, an overall shift in attitude is required if prevailing aquaculture practices are to be changed (Chopin et al., 2008). Society is unlikely to alter established seafood production methods (or any other industry), even if common sense classifies them as unsustainable, unless there are immediate compelling reasons to do so (Chopin et al., 2008). IMTA adoption may require incentives such as a certified eco-labeling system for products or nutrient trading credits (*e.g.* carbon, nitrogen) (Chopin et al., 2012). On the other hand, the notion that consumers will continue to expect more from seafood than simply cost and taste is encouraging for future IMTA endeavours (Neori, 2008). Traceability of food to sustainable fisheries and/or approved aquaculture facilities is increasingly important for

countries importing large quantities of fish, for reasons of public health and customer satisfaction (FAO, 2014).

Environmental Justification for IMTA

As discussed previously, a consequence of typical finfish monoculture is significant loading of both inorganic and organic waste nutrients into the surrounding ecosystem. IMTA aims to convert as much of these wastes as possible into biomass for harvest (Reid et al., 2009). Fed and extractive species selected for an IMTA site should be cultivated in biomass ratios that aim to counterbalance productivity and metabolic processes (Chopin et al., 2007). They should be cultured in proximity, though exact distances between IMTA components are less important than their integration in terms of ecological connectivity, *i.e.* species arrangement should follow nutrient flows to optimize waste nutrient capture and growth (Barrington et al., 2009).

Seaweeds and other aquatic vegetation can aid in absorbing excess dissolved inorganic waste nutrients. While doing so, water quality would be improved for the cultured finfish and surrounding species (Chopin et al., 2001). Macroalgae genera such as *Gracilaria*, *Porphyra*, *Palmaria*, *Chondrus*, and *Laminaria* are efficient nutrient recyclers and are of commercial value in diverse established or developing markets (Chopin et al., 2001). Their cultivation requires low-cost technologies and yields high biomasses (Neori, 2008). Furthermore, seaweeds are fast-growing and can be harvested at different times of the year depending on the species (Chopin et al., 1999). Macroalgae should also not be overlooked as major ingredients in fish and shrimp feed, a more sustainable alternative to wild fish or land-based plant crops (Neori, 2008). Harvested

seaweeds may equate to the removal of tens of metric tonnes of nitrogen from the water, helping to reduce eutrophication, especially in nitrogen surplus systems (*e.g.* Wu et al., 2015). Full remediation of dissolved inorganic nitrogen from finfish culture (or equivalent amount from the surrounding environment) by neighbouring kelp culture is neither practical nor necessary for a successful IMTA system (Reid et al., 2013a).

Organic particulate waste nutrients, the second category of potential food sources in an IMTA system, can be utilized by deposit- or suspension-feeding extractive species. The majority of organic material from salmon farms settles to the ocean floor beneath or nearby the fish cages (Brooks, 2001). Benthic species such as sea cucumbers, polychaetes, and sea urchins can utilize this organic matter and may therefore play an important role in future IMTA development. Their placement is not limited to the benthos where water depth or sediment conditions may not permit optimal growth or survival (Brooks et al., 2003). Instead, deposit feeders could be cultured in suspended infrastructure beneath fish pens (Hannah et al., 2013; Yokoyama, 2013), or even directly inside fish or shellfish cages (Ahlgren, 1998; Zhou et al., 2006).

Small organic particulates that are held in suspension may drift off-site with the current. Several commercially-important suspension-feeders are able to ingest and absorb these high quality (*i.e.* high organic content, Macdonald et al. (2011), Lander et al. (2013)) aquaculture waste particulates from the water column for growth (Handå et al., 2012; Lander et al., 2013; Macdonald et al., 2011; Nelson et al., 2012; Reid et al., 2010). Suspension-feeders should be located based on local particulate dynamics around the fish cages, providing maximum access to this food source (Reid et al., 2010). Filter-feeding bivalve shellfish are the primary group of suspension-feeders being explored for IMTA

and include popular commercial species such as oysters, scallops, and mussels. Bivalves are considered sources of animal protein already well ahead on sustainability criteria as they are grown extensively, filtering seston from the water (Bostock et al., 2010). Phytoplankton are an important constituent of their natural diet though zooplankton are also ingested by a variety of bivalves (Kamiyama, 2011; Lehane and Davenport, 2006, 2002). Omnivory is advantageous under low phytoplankton conditions and could supply the animals with greater energy and result in improved growth (Lehane and Davenport, 2002).

Bivalves Cultured Near Finfish

A number of studies have demonstrated positive impacts of finfish farms on bivalve growth. At an experimental IMTA site in the Bay of Fundy, mussels cultivated in the vicinity of Atlantic salmon farms had a significantly greater feeding activity (measured by exhalant siphon area) than reference mussels a few hundred metres away (Macdonald et al., 2011). Total particulate matter and particulate organic matter were higher at the farms compared to the reference site, which the authors attributed to finfish wastes due to there being no significant differences in natural plankton material (measured by chlorophyll a) (Macdonald et al., 2011). Another study detected only a slight (though significant) increase in the size of mussels suspended at salmon farms when compared with nearby shellfish farms (Stirling and Okumuş, 1995). In this study, mussels grown nearer salmon used significantly less of their energy reserves in the winter (for metabolism and gametogenesis) compared to animals at the shellfish farms, suggesting that the bivalves utilized fish waste nutrients over the winter months (Stirling

and Okumuş, 1995). Pacific oysters were able to recover just over half of the excess organic matter out of the particles in the correct size class, or an estimated 22.65% of the total organic matter (TOM), in the water near sea bass pens (Jiang et al., 2012). The majority of TOM around the pens (54.44%) was nutrients derived directly from the fish (10.33% waste feed, 44.11% faeces), with the remainder of particulates being natural organic seston (Jiang et al., 2012). The instantaneous growth rates of Pacific oysters cultured inside Chinook salmon cages in BC were significantly greater than those of oysters outside of the cages, and both were higher than those of control animals located 4 or 6 km away (Jones and Iwama, 1991).

While some studies link improvements in bivalve growth to finfish wastes, others have shown no significant impact. A field experiment in Australia revealed no significant difference in mussel growth near and away from an Atlantic salmon farm (Cheshuk et al., 2003). The authors report that neither suspended solid waste nor phytoplankton were increased around the farm (Cheshuk et al., 2003). Similarly, Taylor et al. (1992) did not detect nutrient enrichment (seston, chlorophyll a) near two different Chinook salmon farms when compared to their respective control stations 600 or 800 m away, or find any evidence of increased mussel growth closer to the farms (measured at 3 m, 15 m, 75 m, and control). This study has a caveat in that the salmon farms used for the experiment produced only 3700 and 2500 kg of fish (consuming approximately 9250 and 6200 kg of feed, respectively) during the year of study (Taylor et al., 1992). As a comparison, a typical commercial Atlantic salmon farm in BC is licenced for three orders of magnitude more fish biomass (average ~2500 tonnes, DFO, 2015a). The organic suspended particulate “plume” around finfish cages has been theorized, but may only be

intermittently observable in the field (Brager et al., 2015), if at all (Taylor et al., 1992). Sutherland et al. (2001) quantified the suspended particulate matter at a salmon farm in the Broughton Archipelago, BC, with the highest mean concentration 0.6 $mg \cdot L^{-1}$ within the pen during feeding. Approximately 87% of the SPM was observed below the pen, at 20 m depth, whereas 30% of the SPM was observed beside the pen, at 5 m depth, suggesting the movement of suspended particles (predominately fish faecal material) at the site was largely in the vertical direction (Sutherland et al., 2001).

Limitations exist regarding the biomitigative capacity of IMTA bivalves due to the necessity for a horizontal (as opposed to vertical) settling flux of nutrients from fish cages, and time required to intercept these particles, if present (Cranford et al., 2013). Solid organics in the horizontal flux/plume must also be small enough to allow for their filtration by bivalves (Reid et al., 2010). Even if all of the appropriately-sized feed and faecal particles were available to mussels for growth, Wang et al. (2012) argue that the potential yield of mussels would still be low and that natural food sources would remain the most important dietary component. This is echoed by Troell and Norberg (1998) who modelled the output and retention of suspended solids from integrated salmon-mussel culture under different water currents and fish feeding regimes. They concluded that "…the ambient seston concentration is of greater importance in controlling mussel growth, and increases in suspended solids from the fish cages may contribute significantly only during periods of low plankton production" (Troell and Norberg, 1998). Finfish culture indirectly supports mussel growth by discharging dissolved inorganic nitrogen, used for macroalgae production, in quantities that could theoretically support a much greater mussel biomass than nutrients from suspended organics (Wang et

al., 2012). However this indirect use of DIN is spatially expansive and difficult to quantify more specifically than an overall stimulation of natural food webs (Wang et al., 2012).

All extractive components of an IMTA system produce their own wastes. A consequence of requiring shellfish to be cultured very close to finfish cages (to optimally intercept farm-derived suspended organics) is that additional faeces and pseudofaeces from the shellfish may accumulate on the farm tenure and contribute to benthic organic loading (Cranford et al., 2013; Reid et al., 2013b, 2010). Strategic placement of shellfish infrastructure could ameliorate this, though ideally bivalves would consume adequate fish waste material to (at least) compensate for their own wastes (Reid et al., 2013b). For blue mussels feeding on Atlantic salmon solids this threshold value was estimated at 11.5% – 19.6% depending on particle origin (feed fines versus fish faeces), particle size, ambient seston, site hydrology, and other factors (Reid et al., 2013b). IMTA is undoubtedly not a 100 percent efficient bioremediation system and should not be regarded as such, but rather a more balanced alternative to typical fed mono-aquaculture practices (Chopin et al., 2012).

Bivalves as a Biosecurity Tool

Filter-feeding bivalves have been proposed as a potential biomitigation tool for several fish disease agents, and could be strategically cultured at finfish farms to create a "wall" at the interface(s) between farm and wild fish stocks (Chopin et al., 2013). This extractive crop would all the while provide biomitigative and diversification services more typical of IMTA discourses. For example, infectious salmon anaemia virus (ISAV)

is a serious problem for Atlantic salmon farms due to its potential to cause widespread mortality (FAO, 2010). Blue mussels are capable of ingesting and rapidly inactivating ISAV in laboratory experiments (Skår and Mortensen, 2007). All mussels cohabitating with ISA-positive salmon in this study were ISAV negative afterwards, suggesting mussels do not act as a reservoir for this virus (Skår and Mortensen, 2007). Another laboratory experiment showed blue mussels effectively removed harmful phytoplankton from the water when cultured in a holding tank upstream of experimental fish. Compared with the experimental fish, controls exposed to the phytoplankton had double the gill mucous thickness within 3 weeks (Delegrange et al., 2015).

A final example of bivalve filter-feeding exploitation for biosecurity is the removal of planktonic sea lice larvae from the water column as shown in various laboratory experiments (Bartsch et al., 2013; Molloy et al., 2011; Webb et al., 2013). Molloy et al. verified copepodid presence in the buccal cavity and stomach contents of mussels *Mytilus edulis* following 30 and 60 min exposures to the lice (Molloy et al., 2011). All species of shellfish (mussels, Pacific oysters, Pacific scallops, and basket cockles) tested in a series of laboratory experiments were capable of ingesting and digesting sea lice larvae, with and without algae present, with no significant effect of temperature (5, 10, and 15˚C) (Webb et al., 2013). A separate series of laboratory experiments examined bivalve filtration of copepodids under static, flow-through, and recirculating water regimes (Bartsch et al., 2013). Ingestion was successful in all trials and was improved by the addition of light to concentrate the larvae (Bartsch et al., 2013). The aim of this Master's thesis was to continue this laboratory-based research at a larger scale and assess sea lice mitigation by cultured bivalves (Pacific oysters, *Crassostrea*

gigas) at a commercial Atlantic salmon farm in BC. Evidence of louse ingestion and/or population reduction at the farm would expand the potential environmental and social benefits of IMTA. Moreover, the development of alternative, non-chemical louse control techniques will ultimately help decrease the salmon farming industry's reliance on chemical treatments, improving both the environmental performance of salmon farms and their social license to operate.

Research Questions

Pacific oysters were cultured in stacks of Dark Sea trays, spaced 1 m apart, around one end of a commercial Atlantic salmon (*Salmo salar*) farm in BC, as well as at a reference site located approximately 150 m away from the farm. The purpose of this Pacific oyster—Atlantic salmon IMTA was to assess bivalve growth and sea louse mitigation, guided by the following four research questions:

1. H_o: There is no significant difference in oyster growth at the farm versus at the reference site.
H_a: There is a significant difference in oyster growth at the farm versus at the reference site.

2. H_o: There is no significant difference in numbers of parasitic sea lice on salmon in bivalve and non-bivalve fish cages.
H_a: There is a significant difference in numbers of parasitic sea lice on salmon in bivalve and non-bivalve fish cages.

3. H_o: There is no significant difference in the density of planktonic sea lice larvae in bivalve and non-bivalve fish cages.

H_a: There is a significant difference in the density of planktonic sea lice larvae in bivalve and non-bivalve fish cages.

4. H_o: A) There is no evidence of louse ingestion in the oyster digestive tissue. B) There is no significant difference in louse ingestion by oysters on different sides/depths.

H_a: A) There is evidence of louse ingestion in the oyster digestive tissue. B) There is a significant difference in louse ingestion by oysters cultured on different sides/depths.

These research hypotheses are addressed in Chapter 3; first, Chapter 2 will focus solely on describing the sea lice species and stages present using data collected both before and during oyster deployment.

Chapter 2: Planktonic and parasitic sea lice (*Lepeophtheirus salmonis* and *Caligus clemensi*) at a commercial Atlantic salmon (*Salmo salar*) farm in British Columbia

Abstract

Planktonic and parasitic sea lice (*Lepeophtheirus salmonis* and *Caligus clemensi*) were examined at a commercial Atlantic salmon (*Salmo salar*) farm near the Broughton Archipelago, British Columbia (BC) from December 2012 through March 2014. Surface seawater salinity ranged from 19 to 35 PSU. Parasitic sea lice were counted on a minimum of 20 fish in each of three pens per month. Parasite abundance was highest in the winter, peaking in January 2013 at 6.5 lice·fish^{-1} (13.0% *L. salmonis*, 87% *C. clemensi*) and February 2014 at 3.3 lice·fish^{-1} (80.9% *L. salmonis*, 19.1% *C. clemensi*). SLICE® (emamectin benzoate) was administered both winters and rapidly reduced parasitic sea lice numbers. Monthly parasite abundance was significantly correlated with both surface salinity (r^2= 0.28, p=0.04) and sea lice larval (nauplius and copepodid stages) density (r^2= 0.65, p=0.01). Larval density was calculated monthly via triplicate plankton hauls inside of six fish cages, as well as at a reference site approximately 150 m away. Larval density at the farm peaked in January 2013 (mean±SE: 1.28±0.62 m^{-3}) and January 2014 (0.96±0.25 m^{-3}). Sea lice nauplii were found in all samples at the reference site in densities similar to those observed inside of the fish cages. Overall, the majority of sea lice in the plankton samples were nauplii, 87.8%, with copepodids comprising 5.2% and motile stages 1.8%. Surprisingly, the remaining 5.2% of planktonic sea lice were chalimus stages, all of which were identified as *C. clemensi*, and were found both before and after SLICE® administration. For comparison, estimated nauplius densities were

calculated based on established relationships between water temperature and salinity, the number of female lice present, and the approximate number of fish on-site. These estimated densities were significantly (t=3.41, p=0.009) higher than actual nauplius densities observed at the farm. Findings suggest that sea lice larvae were quickly dispersed away from the farm after hatching. This study provides information on all sea lice stages present throughout an Atlantic salmon production cycle in BC (through two SLICE® treatments) and the first report of planktonic chalimus stages of *C. clemensi*, a commercially-relevant though relatively under-studied sea louse species.

Introduction

Within the hundreds of parasitic Caligid copepods known as sea lice, two species are common on both farmed and wild salmonids in British Columbia (BC): *Lepeophtheirus salmonis* and *Caligus clemensi* (Parker and Margolis, 1964). The former is considered a specialist of salmonid fishes (Johnson and Albright, 1992; Pike and Wadsworth, 1999) but more recently was shown to be abundant on threespine sticklebacks (*Gasterosteus aculeatus*) in BC (Jones and Prosperi-Porta, 2011; Jones et al., 2006). In contrast, *C. clemensi* and other members of this genus are generalists, being found on a variety of fish families (Parker and Margolis, 1964). All caligid copepods possess a free-swimming larval phase which generally consists of two nauplius stages followed by one infective copepodid stage. From one mating event an adult female *L. salmonis* may produce more than 10 sets of egg strings, extruded in pairs, each pair having 200 or more eggs (Heuch et al., 2000). *Caligus clemensi* females produce fewer than 200 eggs at a time (Johnson and Jones, 2015). Louse development through the

various life stages is temperature-dependent (Heuch et al., 2000; Johnson and Albright, 1991) and requires a salinity of 30 PSU or higher for copepodids to be capable of successful host infection (Bricknell et al., 2006; Johnson and Albright, 1991). The first nauplius moults within hours to days into the second nauplius which is anatomically similar but with a slightly longer, more tapered body. In 1.5 to 7 days the second nauplius will moult into a copepodid (Johnson and Albright, 1991), with nauplii perhaps seeking warmer sections of the water column to expedite this process (Norði et al., 2015). Attachment of copepodids onto a fish host marks the transition into the parasitic phase consisting of chalimus, pre-adult, and reproductive adult stages. Pre-adult and adult stages are collectively referred to as motiles whose feeding on fish skin, mucus, and blood is associated with negative economic, environmental, and social consequences (Johnson et al., 2004).

The salmon farming industry in BC has been criticised for the notion that infections on farmed Atlantic salmon may increase sea lice infections on wild Pacific salmonids (*Oncorhychus* spp.) leading to mortality beyond natural levels, in particular juvenile pink salmon (*Oncorhychus gorbuscha*) (Krkošek et al., 2007, 2006). These fish are considered especially vulnerable to sea lice due to their small size (0.2 g, Brauner et al., 2012) during the outmigration from rivers to the Pacific Ocean (Krkošek, 2009; Morton et al., 2005) and coinciding underdeveloped immune (Finstad et al., 2000; Jones et al., 2008; Sutherland et al., 2011), ionoregulatory (Brauner et al., 2012), and osmoregulatory (Sackville et al., 2011) systems that are important for louse resistance. Precautionary measures to minimize potential impacts are a central driver of current sea lice management policies in BC (Saksida et al., 2011b). These policies have been

seemingly successful at reducing sea lice epizootics on wild fish over the last decade through the productive debate and discussion of this issue among scientists, various stakeholders, and policy-makers (Peacock et al., 2013). At salmon farms in BC "a management action" is required (*i.e.* farms must either be treated or harvested) if the mean abundance of motile *L. salmonis* exceeds 3 per fish during the juvenile pink salmon migration from March until July (Saksida et al., 2011b). The only approved sea lice treatment in BC until 2014 was an avermectin in-feed chemotherapeutant formulation called SLICE® (active ingredient emamectin benzoate) (Burridge and van Geest, 2014). Overall, lice in BC do not appear resistant to SLICE® although bioassays have demonstrated significant differences in efficacy of the drug between and within farms (Saksida et al., 2013). A commercial formulation of hydrogen peroxide (Paramove 50™) has recently been approved and administered in certain parts of the province (Morrison, 2014).

The ability of larvae to move among farmed and wild fish populations is largely dictated by local hydrology and is ultimately limited by the longevity of these endogenously-feeding stages. Dispersion of sea lice larvae in the marine environment has been modeled for several salmon farming regions around the world, incorporating a multitude of biotic and abiotic factors (*e.g.* Kristoffersen et al., 2014; Murray and Gillibrand, 2006; Stucchi et al., 2011). Observational data with which to validate models may be limited or absent as larvae are rapidly diluted and typically less detectable away from farms (Costelloe et al., 1996; Penston et al., 2008a, 2004). Sea lice larvae that have been recovered have, for the most part, come from surface plankton tows in densities lower than 1 m^{-3} (Costelloe et al., 1998; McBeath et al., 2006; Norði et al., 2015; Penston

et al., 2008b, 2004). A Scottish study reported exceptionally high average larval density values at an off-shore site (peaking at >500 m^{-3}), demonstrating that sea lice can be found in high densities away from salmon farms under certain conditions (Penston et al., 2004).

McKibben and Hay (2004) concluded that sea lice larvae found in plankton samples originated from salmon farms nearly 5 km away, as no larvae were found when farm gravid lice numbers were zero. Other papers have similarly reported significant correlations between copepodids observed in the water column and numbers of gravid *L. salmonis* at the nearest farm source(s) (Penston and Davies, 2009; Penston et al., 2008b). The relocation of a salmon farm in Scotland resulted in a significant decrease in larval density at the site though copepodid density did not decrease significantly, presumably because this stage continued to be transported to the site from other sources ≥5 km away (Penston et al., 2011). In the same area of Scotland, wind-driven currents were hypothesized to play an important role in louse dispersion; wind data (along with other variables) were used to model dispersion and visualize risk distribution (Murray and Gillibrand, 2006). Wind was an important factor affecting *L. salmonis* copepodid transport in the Faroe Islands, as the infective larvae in this field study were observed where the predominant winds moved surface waters towards the shore (Norði et al., 2015). Dispersion distance of infective copepodids is a motivation for coordinated sea lice management in an area to lessen re-infection of treated sites within range (McKibben and Hay, 2004).

In BC, sea lice are typically lowest in concentration during the summer, with numbers increasing in the fall (Saksida et al., 2007a, 2007b) and peaking by around February in the Broughton Archipelago (Beamish et al., 2006; Orr, 2007). Previous

studies have estimated the egg and larval production from farms in the Broughton Archipelago (Orr, 2007; Stucchi et al., 2011), the most intensively-farmed salmon aquaculture area in BC (Brewer-Dalton et al., 2015) and one of the most complex aquaculture regions in the world with respect to hydrodynamic regime (Foreman et al., 2015). Stucchi and colleagues (2011) reported an average of 580 eggs per gravid female at active salmon farms in this area. They used this egg production data together with water circulation, temperature, and salinity to estimate copepodid abundance (Stucchi et al., 2011). Predicted levels of the infective larvae were usually less than 0.1 m^{-3} and lower than field observations in the Broughton Archipelago (Stucchi et al., 2011). Areas with very low predicted levels of copepodids coincided with both no copepodids found in plankton samples as well as an absence of lice on wild juvenile salmon (Stucchi et al., 2011).

Further understanding of sea lice abundance at, and dispersal away from, farms may help focus louse mitigation efforts (*e.g.* monitoring, treatment, fallowing) to high-risk areas and inform aquaculture policies that lessen lice-related risks to wild fish stocks (*e.g.* strategic siting of new salmon farm tenures, creation of sea lice management zones) (Foreman et al., 2015). This study describes the planktonic and parasitic sea lice present at a commercial Atlantic salmon farm in BC from December 2012 through March 2014. Seasonal patterns, louse species composition, SLICE® treatment effects, and relationships between parasite abundance and larval densities are discussed.

Methods

Study Site

The study was conducted at a Grieg Seafood BC Ltd. commercial Atlantic salmon farm near Turnour Island, BC (50˚36.5' N, 126˚21.9' W), south of the group of islands known as the Broughton Archipelago (Figure 3). Samples were collected between December 2012 and March 2014. The site was stocked in April 2012 and was operational for the duration of this study.

Planktonic Sea Lice

Plankton samples were collected using a 150-µm mesh plankton net (0.5-m diameter x 1.5-m long, Dynamic Aqua-Supply Ltd., Surrey, BC). Monthly vertical plankton hauls were performed inside of six Atlantic salmon pens, in triplicate, from December 2012 through March 2013, and again from September 2013 through March 2014. Samples were not collected from April 2013 through August 2013. The two sea lice sampling periods represent distinct sea lice infection periods, separated by a SLICE® treatment. For three months (January through March 2014), one triplicate was also collected from a reference site which was an empty holding pen approximately 150 m away from the farm. Plankton hauls were performed within an hour of high tide over 2–3 days each month. To sample, the plankton net with an attached flow meter (General Oceanics, Miami, FL) was lowered slowly to the bottom of the pen, approximately 18 m. The net was then hauled to the surface at approximately 0.5 $m \cdot s^{-1}$. Once at the surface, the flow meter reading was recorded and the net was immediately rinsed from the outside using seawater in a manual pump garden sprayer. The sample was concentrated into the

net's cod end and then rinsed into one or more bottles containing 10% formaldehyde, buffered using 1-µm filtered seawater. Fixed samples were brought back to the laboratory for analysis.

Preserved samples were carefully rinsed with freshwater in a 150-µm sieve under a fumehood to remove formaldehyde. Plankton raceways were used to examine the samples, in full, for sea lice under a dissecting microscope (SMZ1000, Nikon, Tokyo, Japan). All sea lice stages were identified and counted. Note that planktonic sea lice will be used to refer to any louse stages found in the plankton sample whereas larval sea lice will refer only to nauplii and copepodids (Figure 4). Sea lice nauplii were not identified to stage (1 or 2) or to species, but were likely *L. salmonis* or *C. clemensi* as later stages of both species were present at the farm throughout the experiment (see Discussion). Once complete, the samples were rinsed into 70% ethanol for long-term storage. Identified sea lice were removed and stored separately in 70% ethanol with any others from the same sample. Two samples per month were later randomly for re-analysis to estimate larval recovery error (Costelloe et al., 1998). Larval densities were calculated as the number of nauplii and copepodids in the sample divided by the volume of water filtered, calculated according to the flow meter manufacturer's instructions.

Attached Sea Lice

Parasitic sea lice were counted on the fish monthly (by farm staff and/or A. Byrne) in accordance with mandatory sea lice monitoring protocols (Saksida et al., 2011b). Each month, 20 fish were randomly selected from each of three pens (one reference and two others) for lice counts. When logistically possible, lice on fish from

additional pens were counted for the sake of this study. Fish were anaesthetized in MS-222 before counting the following categories of sea lice: chalimus (no species distinction), *L. salmonis* pre-adult, adult male, adult female, and gravid female, and motile *C. clemensi* (no further stage separation for this species). Lice that became detached inside of the tote were also recorded after each pen was sampled. In this study parasite abundance (or mean parasite abundance) refers to the number (or mean number) of parasitic lice per fish sampled (Margolis et al., 2013).

Water Temperature and Salinity

From December 2012 until February 2013, water temperature at 1 m was measured using a YSI 30 (HydroScientific West, Poway, CA). Beginning in July 2013 and for the remainder of the study, water temperature was recorded every 30 min by TidbiT® v2 temperature loggers (Onset Computer Corporation, Bourne, MA) deployed at 1, 6, and 18 m depths at the farm. Data were offloaded every few months using a HOBO® shuttle and software (Onset Computer Corporation). Water salinity was recorded once daily by the farmers at 5, 10, and 15 m depths.

Estimated Nauplius Density

Theoretical nauplius densities were calculated for each month that nauplii were counted, using Equations 1—3 in Stucchi et al. (2011) and dividing by the total volume of the farm contained within the pens, based on dimensions of 210 m long x 60 m wide x 18 m deep. Net pens at the site were 30 x 30 m, arranged in a 2x7 array. Depth was set at

18 m, which was the starting depth of all plankton hauls and the approximate depth of the net pens. The rate of viable egg production, P_E, was calculated using Equation 1

Equation 1: $$\boldsymbol{P_E = \frac{1}{\tau_s} \cdot E_S \cdot \rho_E}$$

where τ_s is the hatching time of the first egg on the string, E_s is the number of eggs in a pair, and ρ_E is the proportion of eggs that develop into active nauplii, estimated here as 0.55 which fits the salinities observed in this study (Stucchi et al., 2011). The hatching time of the first egg on the string, τ_s, was calculated using Equation 2

Equation 2: $$\boldsymbol{\tau_s = \left[\frac{\beta_1}{T-10+\beta_1\beta_2}\right]^2}$$

where T is temperature in degrees Celsius and β_1 and β_2 are parameters 41.98˚C $d^{-0.5}$ and 0.338˚C $d^{-0.5}$, respectively, computed by Stein et al. (2005). The total daily production of active nauplii from the farm, T_N, was then calculated using Equation 3

Equation 3: $$\boldsymbol{T_N = P_E \cdot C_{AF} \cdot N_{Fish}}$$

where P_E is the production rate of active nauplii, C_{AF} is the average number of adult female lice per fish, and N_{Fish} is the number of fish at the farm. Finally, nauplius density was calculated by dividing the total nauplii, T_N, by the estimated volume of the farm lease contained within the pens (Equation 4)

Equation 4: $\boldsymbol{Total\ volume\ of\ farm\ lease\ occupied\ by\ pens = l \times w \times d}$

where total l (length), w (width), and d (depth) were set at 210, 60, and 18 m, respectively. The calculation was later re-run using gravid female *L. salmonis* numbers, rather than adult female *L. salmonis* (which includes gravid and non-gravid adults), as the latter may over-estimate nauplius densities at the farm.

Observed nauplius densities were calculated using only plankton data from the farm, not the reference site, and were slightly lower than larval densities presented due to the exclusion of copepodids. Average monthly water salinity and temperature was calculated by averaging daily measurements from 5 and 6 m, respectively.

Statistical Analyses

Statistical analyses were conducted using JMP® 12 (SAS® Institute Inc., Cary, NC). Monthly average larval densities were calculated by taking the average of the pen means (each pen triplicate was considered one independent point). The relationships between parasite abundance and salinity and parasite abundance/adult female louse abundance and larval density were assessed using pairwise correlation. Observed and estimated densities of nauplii were compared using a Student's paired t-test. Statistical significance was reported when $p<0.05$

Results

Study Site

The study site was initially stocked in April 2012 and harvest was completed by June, 2014 (re-stocked with new fish in July 2014). Monthly average water temperature during the experiment, December 2012 through March 2014, was 6.9—16.7°C at the surface, 6.9—10.5°C at 6 m depth, and 6.8—8.9°C at 18 m depth. Monthly average water salinity from December 2012 through March 2014 was 19—34 PSU at the surface, 24—34 PSU at 5 m depth, 30—34 PSU at 10 m depth, and 31—34 PSU at 15 m depth. The lowest salinity measurements occurred in August (Figure 5).

Planktonic Sea Lice

On average, 2.73±0.04 m^3 (mean±SE) of seawater was filtered per plankton haul. Larval density peaked in January both in 2013 (1.28 larvae·m^{-3}) and 2014 (0.96 larvae·m^{-3}) (Table 1). The majority of planktonic sea lice found in samples were nauplii both in 2012–2013 (73.9%) and 2013–2014 (93.3%) (Table 2). All sea lice found at the reference site were nauplii. March 2013 samples were collected, but not analyzed due to their large size and the lack of attached sea lice at the farm at that time. Plankton sampling resumed in September 2013, though no free-living sea lice stages were found until October 2013. From December 2012 through February 2013, 38 of 54 samples contained sea lice (70.4% positive) and from October 2013 through March 2014, 90 of 123 samples contained lice (73.2% positive). On two occasions, an Atlantic salmon was caught in the

plankton net; chalimus and any other attached stages in these samples were not used in overall planktonic sea lice counts.

Two samples per month from October 2013 to March 2014 (12 total) were selected randomly and re-examined in full for sea lice which may have been missed. Sea lice were observed in one of these samples (two nauplii), giving a larval recovery error (Costelloe et al., 1998) of ± 0.17. A total of 17 chalimus stages were found in the plankton samples in December 2012 (5), February 2013 (8), December 2013 (2), and February 2014 (2) (Table 2). All chalimi were identified as *C. clemensi* (Figure 6) (Johnson and Jones, 2015; Kabata, 1972).

Estimated Nauplius Density

Estimated nauplius densities were calculated based on the number of adult female *L. salmonis* per fish, water temperature and salinity, the approximate number of fish on-site, and the "volume" of the farm. The total number of fish used in the calculations, 700,000, is equivalent to 50,000 fish per pen or 0.15 $kg \cdot m^{-3}$ based on the farm volume used and fish weighing 5 kg each. Resulting estimated nauplius densities were significantly higher than those observed ($t=3.41$, $p=0.009$). When gravid female *L. salmonis* numbers were used (rather than all adult females, gravid and non-gravid) the resulting estimated densities were lower, on average by 50%, though still significantly higher than observed densities ($t=3.17$, $p=0.013$) (Table 3).

Attached Sea Lice

Overall, sea lice populations differed in the quantity and the dominant species present between the two sea lice "seasons", *i.e.* winters, sampled. For the period December 2012 through February 2013, mean parasite abundance peaked at 6.5 lice·fish^{-1} in January and on average motile stages were 19.7% *L. salmonis* and 80.3% *C. clemensi* (Table 4). In contrast, for the period December 2013 through February 2014, peak parasite abundance was 3.3 lice·fish^{-1} in February, and on average, *L. salmonis* motiles increased to 94.6% (Table 4). Over the course of the study, 36 of the 45 highest lice counts on individual fish (80%) were observed in January 2013, during the *C. clemensi*-dominated infection period. The highest recorded count was 27 lice·fish^{-1}, also in January 2013 (see Appendix Table 10 for raw lice count data).

No lice counts were performed in October 2013 due to low dissolved oxygen at the site that would have caused unwarranted fish stress and mortality. Two SLICE® treatments were administered during the study, the first in January 2013 and the second in February 2014 (Figure 7). Within one month of SLICE® administration, numbers of attached sea lice stages dropped by >60% both years (Figure 7).

Mean parasite abundance (all parasitic stages of both species) was strongly correlated with sea lice larval density (r^2= 0.65, p=0.01) (Figure 7, Figure 8). Adult female *L. salmonis* abundance alone was not significantly correlated with larval density (r^2= 0.23, p=0.55). Mean parasite abundance was also correlated with salinity at 1 m depth (r^2= 0.28, p=0.04) which, out of daily salinity measurements at 1, 5, 10, and 15 m, showed the greatest fluctuation between winter (>30 PSU) and summer (<20 PSU) salinities (Figure 5, Figure 8).

Discussion

Planktonic Sea Lice

Vertical plankton hauls performed in this study generally filtered less than 3 m^3 of water – one or more orders of magnitude less volume than other studies that used boat-drawn plankton nets (Jackson et al., 1994; McKibben and Hay, 2004; Norði et al., 2015; Penston et al., 2011, 2008b). Nevertheless, sea lice were found in the majority of plankton samples. Nauplii were presumably a mixture of *L. salmonis* and *C. clemensi*, the two species of sea lice found on the farm salmon; however, sea lice nauplii are difficult to identify to species and it cannot be ruled out that these belonged to different species of lice that did not originate from the farm. Plankton samples were preserved in formalin, thus genetic species verification was not possible. Nauplius stages were present in all nine samples (range 1—4 larvae·$sample^{-1}$) collected from the reference site, in densities similar to those taken from inside the salmon cages (Table 1). No other sea lice stages were observed at the reference site. Assuming the nauplii derived from infections of the farm, this suggested that the planktonic stages quickly dispersed from the farm and, given that it takes several days for nauplii to develop into copepodids, it is not surprising that copepodids were a rarity in the plankton samples, comprising only about 5% of the planktonic sea lice found. Stucchi and colleagues noted that, in general, their modeled copepodid concentrations for the Broughton Archipelago were low (usually <0.1 m^{-3}) (Stucchi et al., 2011).

Estimated nauplius densities based on adult female or gravid *L. salmonis*, water temperature and salinity, and the estimated number of fish on-site were found to be significantly higher than the observed densities; using gravid *L. salmonis* numbers in the

calculation did result in lower nauplius densities that were closer to the observed values, though still significantly higher (Table 3). It is suggested that other studies using the formulae developed by Stucchi et al. (2011) and Stein et al. (2005) consider the same approach of using gravid female count data, if available, to potentially obtain a more accurate estimate. As nauplii are not yet infective, copepodid abundance in the water column may provide a more useful metric and predictor of sea louse transmission rates (Tully and Whelan, 1993). Copepodid densities at the farm were low and consequently so was the infective pressure of sea lice larvae at this location (though not all copepodids are infective), consistent with the low copepodid densities (maximum 0.28 copepodids·$m^{-3)}$ observed by Costelloe et al. (1996) inside of salmon cages in Ireland. In addition to louse infection via copepodid larvae, motile sea lice stages are known to jump hosts (Hull, 1998; Tully and Nolan, 2002) and six were observed in plankton samples in this study, four *C. clemensi* and two *L. salmonis* (1.8% of all planktonic sea lice found). Overall, the data support a strong relationship between planktonic and parasitic louse stages at the farm both visually (Figure 7) and statistically (r^2= 0.65, p=0.01) (Figure 8).

Planktonic Chalimus Stages

A total of 17 detached chalimus stages were found in the plankton samples, all of which were identified as *C. clemensi* (Figure 6, Table 2) (Johnson and Jones, 2015; Kabata, 1972). This was surprising as chalimi are immobile, anchored to the fish via their frontal filament until the next phase of the life cycle; the majority of the chalimi recovered in this study still possessed at least a partial frontal filament. To the author's knowledge, no other studies have reported chalimus stages in plankton samples. In 2012–

2013, five of the 13 chalimi were found prior to SLICE® administration, and in 2013–2014, two of the four chalimi were found prior to treatment with SLICE®.

In one plankton sample, from December 2013, a fish was caught in the net and later inspection of the sample revealed 11 chalimi. These were presumably off the fish and were not counted towards the planktonic lice numbers presented in this paper. Because this was accidental and the fish was not anaesthetized, the captured salmon was simply rinsed with filtered seawater and returned to the pen as quickly as possible (no detailed inspection for attached louse stages was performed). The rinsing process likely facilitated chalimus removal. Interestingly, only copepodid and motile stages are considered subject to loss due to capture and handling (Johnson and Jones, 2015). As well, no chalimi were recorded during lice counts on any of the 60 fish counted that month, indicating either this fish was anomalous (possible, given that a fish getting caught in the plankton net was extremely rare) and/or the small chalimus stages were missed during routine farm lice counts.

Attached Sea Lice and Temporal Effects

Lepeophtheirus salmonis infections ordinarily increase with ocean residency time (Saksida et al., 2015) whereas *C. clemensi* are typically found in higher abundance on younger salmon (Saksida et al., 2011a). Both of these findings held true in the present study wherein the first year, 2012–2013, attached sea lice counts were dominated by the more transient *C. clemensi* and the following year showed increased numbers of *L. salmonis* and substantially decreased *C. clemensi* prevalence (Table 4). Sea lice abundance peaked in this study in January 2013 and February 2014 and SLICE® was

administered during both peaks. The month after SLICE® administration, attached sea lice were reduced by >60% in both years, and numbers continued to drop in subsequent monthly counts (Figure 7). These treatments were within the timeframe suggested by Rogers et al. (2013) and Krkošek et al. (2010) as being the optimal SLICE® administration window to supress farm sea lice numbers in the Broughton Archipelago during the March – June juvenile Pacific salmon migration.

Figure 7 shows a clear seasonal pattern, characteristic of BC, where parasite abundance is highest in the winter and drops off during the summer months. Larval densities mimicked this seasonal pattern and it has previously been estimated that *L. salmonis* egg production in the Broughton Archipelago is highest in November–December (Orr, 2007). Increased sea lice at salmon farms through the fall/winter could be attributed to BC's large wild salmon populations returning to their natal rivers to spawn (Beamish et al., 2005). The spring/summer drop in parasite abundance is associated with chemical sea lice treatments administered in the spring, in anticipation of wild juvenile salmon migration (Peacock et al., 2013). Natural salinity declines may play a role in keeping lice counts low during the summer months in BC (Brooks and Stucchi, 2005), particularly for *C. clemensi* which is perhaps less tolerant to low salinity than *L. salmonis* (Jones et al., 2006). Attached sea lice numbers were significantly correlated with average surface (1 m) salinity which showed the most drastic changes, ranging from >30 PSU in the winter to <20 PSU in August (Figure 5).

Summary and Conclusions

This study examined planktonic and parasitic sea lice at a commercial salmon farm over the course of two infection periods in the winters of 2012–2013 and 2013–2014. During this period, parasite abundance followed a seasonal pattern and peaked in January 2013 and February 2014. The decline following both peaks can be attributed to SLICE® treatments. Monthly parasite abundance was significantly correlated with both surface salinity (r^2= 0.28, p=0.04) and sea lice larval density (r^2= 0.65, p=0.01). Sea lice larvae (mostly nauplii) were found in the majority of plankton samples taken inside of the fish cages and nauplii (only) were found in all plankton samples collected at a reference site approximately 150 m away from the farm. Nauplius densities in the farm samples were significantly lower than estimated densities. Together these findings suggest that sea lice nauplii quickly dispersed from the farm after hatching. Whether this dispersion is random or aggregated at a broader scale is an important area of further research on farm-derived sea lice copepodids. The discovery of planktonic *C. clemensi* chalimi warrants further investigation into this species' behaviour in the field and whether they are capable of re-infecting a host once detached. Given the concerns in BC surrounding farm placement and wild salmon migration corridors, it is valuable to know how many infective stages of sea lice are actually present in the water column at farm sites to better assess the risk for wild salmon swimming in close proximity.

Chapter 3: Pacific oyster (*Crassostrea gigas*) growth and sea lice mitigation at a commercial Atlantic salmon (*Salmo salar*) farm in British Columbia

Abstract

The integrated culture of bivalves at marine finfish farms may help lessen the negative effects of organic waste nutrient loading from the finfish, while producing a second commercially-valuable crop and increasing the farm's overall social license to operate. An added benefit of culturing filter-feeding bivalves at salmon farms may be the natural mitigation of planktonic sea lice larvae. This study assessed the growth of Pacific oysters (*Crassostrea gigas*) cultured at a commercial Atlantic salmon (*Salmo salar*) farm in British Columbia (BC) and the extent to which the salmon louse, *Lepeophtheirus salmonis*, was mitigated. Oysters were deployed in Dark Sea trays at 1, 3, and 6 m depths around one end of the farm and at a reference site approximately 150 m away. Oyster growth measurements included shell height, length, and width; whole wet weight; and soft tissue wet, dry, and ash-free dry (organic) weight. All seven variables increased significantly over time (though most did not increase significantly between November 2013 and March 2014) with significant effects of depth and position around the farm ("side", which includes the reference site). In general, oysters at 1 and 3 m were significantly larger than those at 6 m. Side of the fish cage was used as a blocking factor in the experimental design and had a significant effect on final oyster size; at the end of the study, oysters at the farm were either significantly larger or not significantly different than oysters at the reference site, depending on the side of deployment. Sea lice mitigation was assessed monthly by comparing the water-borne density of larval sea lice

among the three bivalve cages and three control (non-bivalve cages). There was no significant variation in mean larval density due to time ($F_{5,24}$=0.86, p=0.52) or treatment ($F_{1,24}$=2.03, p=0.17). Larval densities were highest in January, 2014 and oyster digestive tissues preserved this month (n=162, encompassing all sides/depths) were analyzed for evidence of louse ingestion by examining for *L. salmonis* DNA. Sea lice DNA was not detected in these samples.

Introduction

Integrated Multi-Trophic Aquaculture

Integrated multi-trophic aquaculture (IMTA) can be defined as the farming of aquaculture species from different trophic levels, whereby one species' waste nutrients are recaptured into energy for others, mimicking synergistic relationships found in natural ecosystems (Chopin et al., 2012). In this way IMTA farms produce multiple crops of seafood, theoretically increasing the overall economic and ecological efficiency of a site. The IMTA concept has been practiced for centuries in Asia (*e.g.* China, Chan (1993)), and it stands in contrast to the monoculture crops typical of Canadian aquaculture (Chopin, 2015) and Western farming in general.

As with any animal production system, net pen salmon farms directly impact the surrounding environment to varying degrees, for instance through enrichment from both dissolved inorganic (*e.g.* nitrogen and phosphorus) and settled or suspended organic (*e.g.* excess fish food, faeces) wastes. These dissolved inorganic, settled organic, and suspended organic waste nutrients could be captured and utilized for growth by IMTA extractive species such as kelps, sea cucumbers, and mussels, respectively.

Most of the organic waste material and suspended particulates from salmon farms accumulates on the ocean floor beneath the site (Brooks, 2001; Sutherland et al., 2001), though smaller organic particulates held in suspension are observed (to a lesser degree) directly beside fish pens (Brager et al., 2015; Sutherland et al., 2001). The prolonged and concentrated build-up of organic waste material on the ocean floor can alter natural benthic processes (Brooks and Mahnken, 2003; Brooks et al., 2003; Kutti et al., 2008, 2007), potentially reducing benthic biodiversity (*i.e.* few opportunistic species in high abundances, Pearson and Rosenberg (1978)) and/or creating azoic sediment zones (Findlay and Watling, 1997). Fallowing of farm tenures allows for biological and chemical remediation of the benthos which generally occurs within six months (Brooks et al., 2003). In Canada, benthic monitoring during finfish production and benthic remediation between production cycles is assessed by a variety of biological and chemical criteria in accordance with the federal Benthic Monitoring Program (DFO, 2013). Organic extractive species cultured beneath or in close proximity to fish cages may help capture a portion of these waste nutrients, diverting them instead towards the growth of commercially-valuable products.

Finfish – Bivalve Integrated Multi-Trophic Aquaculture

Several studies have demonstrated positive impacts of finfish farms on the growth of filter-feeding bivalves such as mussels (Macdonald et al., 2011; Stirling and Okumuş, 1995) and oysters (Jiang et al., 2012; Jones and Iwama, 1991) cultured nearby. However, studies such as those by Cheshuk et al. (2003) and Taylor et al. (1992) reported no significant increase in mussel growth near finfish cages. Conflicting results are not

entirely surprising as the suspended organic particulate "plume" around finfish cages, while logical, may only intermittently occur (Brager et al., 2015) if at all (Taylor et al., 1992) depending on the site. As well, these studies varied with respect to fish species and quantity and the distance of reference sites. For example, Taylor et al. (1992) examined mussel growth near Chinook salmon farms in BC, compared to reference sites 600 and 800 m away, and did not detect nutrient enrichment (seston, chlorophyll a) or find any evidence of increased mussel growth closer to the farms (measured at 3 m, 15 m, 75 m, and control). However the salmon farms in this study produced only 3700 and 2500 kg of fish (consuming approximately 9250 and 6200 kg of feed, respectively) during the year of study (Taylor et al., 1992). As a comparison, a commercial Atlantic salmon farm in Canada contains three orders of magnitude more fish biomass (DFO, 2015a) and would presumably create a greater suspended organic nutrient plume available to cultured bivalves. Jones and Iwama (1991) concluded that the instantaneous growth rates of Pacific oysters were significantly higher inside of commercial-scale Chinook salmon cages, compared to control animals outside of the cages and at reference sites 4 or 6 km away.

For effective bivalve-finfish IMTA, solid organics in the horizontal flux/plume must be present and small enough to allow for their filtration by bivalves (Cranford et al., 2013; Reid et al., 2010). Even if all of the appropriately-sized feed and faecal particles were available to mussels for growth, natural food sources (ambient seston) would likely remain the most important dietary component for mussels (Troell and Norberg, 1998; Wang et al., 2012). It can be concluded that shellfish must be cultured very close to finfish cages to optimally intercept farm-derived suspended organics. This means,

however, that faeces and pseudofaeces from the shellfish crop may accumulate on the farm's tenure and contribute to benthic organic loading (Cranford et al., 2013; Reid et al., 2013b, 2010). Strategic placement of shellfish infrastructure could ameliorate this, though ideally bivalves would consume adequate fish waste material to compensate for their own wastes (Reid et al., 2013b). IMTA is undoubtedly not a 100 percent efficient bioremediation system and should not be regarded as such, but rather a more balanced alternative to typical fed mono-aquaculture practices (Chopin et al., 2012).

Sea Lice at Salmon Farms in British Columbia

Salmon farms may also impact the surrounding ecosystem through the acquisition, amplification, and spill-back (to wild fish) of pathogens. Indeed, much of the criticism towards the salmon farming industry in BC relates to allegations of farm salmon harming the health of native Pacific salmonids (*Oncorhynchus* spp.). Sea lice (ectoparasitic copepods of the family Caligidae) are one of the more well-known examples. Infestations of these parasites are more likely at high host densities, such as those found at salmon farms, and can result in economic losses from reduced fish growth, feed conversion, and market value; treatment costs; and direct or indirect mortality (Johnson et al., 2004). In BC, two species of sea lice commonly parasitize farm and wild salmonids: *Lepeophtheirus salmonis* and *Caligus clemensi*. The latter are smaller and less damaging to the fish host and can be found on a variety of fish families (Parker and Margolis, 1964) compared to *L. salmonis*, the salmonid specialist (Tully and Nolan, 2002).

Atlantic salmon farms in BC have been accused of increasing sea lice infections on wild salmonids, in particular juvenile pink salmon, leading to higher mortality rates than would be seen naturally (Krkošek et al., 2007, 2006). Farm salmon are systematically monitored at least monthly in BC for sea lice infections (details of which are available to the public on the Fisheries and Oceans Canada website). Treatment or other management action is taken if a threshold level of three motile stages per fish is reached from March through June, during the migration of pink and chum salmon smolts from rivers into the Pacific Ocean. Pink salmon are relatively small upon entry to the Pacific Ocean (0.2 g, Brauner et al., 2012) and appear to be at the greatest risk of sea lice-induced mortality until they reach 0.7 g (Jones et al., 2008).

The sea louse life cycle begins with a free-swimming larval phase comprised of two naupliar stages followed by one infective copepodid stage. A single salmon farm experiencing lice infestation can add millions of sea lice nauplii into the surrounding environment each day (Orr, 2007; Tully and Whelan, 1993). Sea lice larval movement is largely dictated by local hydrology (Tully and Nolan, 2002) though both nauplii and copepodids are capable of swimming and respond to gradients of light (copepodids, Heuch et al., 1995), salinity (copepodids, Heuch, 1995), or temperature (nauplii, Norði et al., 2015). Field studies suggest that planktonic sea lice accumulate in shallow water (Costelloe et al., 1996; McKibben and Hay, 2004; Norði et al., 2015; Penston et al., 2011, 2004) and farmed salmon held at shallower depths (0—4 m) evidently develop significantly greater sea lice infections than those held at greater depths (4—12 m) (Hevrøy et al., 2003). *Lepeophtheirus salmonis* copepodids are well-equipped with a variety of sensory organs (*e.g.* eyespots, mechanical and chemical receptors) to target a

salmonid host (Bron et al., 1993). Once attached to a suitable host, copepodids progress through the remaining chalimus, pre-adult, and adult life stages.

IMTA Bivalves and Sea Lice

In addition to recycling small organic particulate wastes at salmon farms, filter-feeding bivalves could be strategically cultured at finfish farms to create a "wall" at the interface(s) between farm and wild fish stocks (Chopin et al., 2013) and have been proposed as a potential biomitigation tool for several fish disease agents such as infectious salmon anaemia virus (ISAV) (Skår and Mortensen, 2007) or harmful phytoplankton (Delegrange et al., 2015). The ability of bivalves to ingest and digest sea lice larvae in the laboratory has been well documented. Molloy et al. (2011) verified copepodid presence in the buccal cavity and stomach contents of a laboratory population of blue mussels (*Mytilus edulis*) following 30 and 60 min exposures to the lice. Similarly, all species of shellfish tested (mussels, Pacific oysters, Pacific scallops, and basket cockles) in a series of laboratory experiments ingested and digested sea lice larvae, with and without algae present, with no significant effect of temperature (5, 10, and 15˚C) (Webb et al., 2013). A separate series of laboratory experiments examined bivalve filtration of copepodids under static, flow-through, and recirculating water regimes (Bartsch et al., 2013). Ingestion was successful in all trials and was improved by the addition of light to concentrate the larvae (Bartsch et al., 2013). Evidence of natural sea louse mitigation by cultured bivalves in the field would expand the potential environmental and social benefits of IMTA and encourage further development of

alternative, non-chemical, sea louse mitigation strategies. The present study examined the growth of, and sea louse mitigation by, Pacific oysters cultured at a commercial Atlantic salmon farm in BC.

Methods

Study Site

The study was performed at a Grieg Seafood BC Ltd. commercial Atlantic salmon farm off of Turnour Island, BC, Canada (50˚36.5' N, 126˚21.9' W). Samples were collected between September 2013 and August 2014. The site was stocked in April 2012 and harvested by June 2014 (re-stocked in July 2014).

Oyster Deployment

Large seed Pacific oysters (*Crassostrea gigas*, mean shell height±SE 81.1±0.9 mm) were purchased in July 2013 from Mac's Oysters Ltd. (Fanny Bay, BC) in Dark Sea trays (dimensions approximately 60 x 60 x 8 cm with mesh size 12 mm x 12 mm). They were transported to the salmon farm in a refrigerated (10°C) truck and then on a ferry (approximately 10 h total transportation time), covered with seawater-soaked blankets and sprayed with seawater periodically to prevent desiccation until deployment. One stack of oyster trays was deployed every 1 m systematically at 1, 3, and 6 m depths (from the surface to the top of the stack) around 5 outer walkways of the fish cage array (designated as sides A–E), as well as at a reference site approximately 150 m away from the farm (designated as side F) (Figure 9). Each stack was comprised of four Dark Sea

trays: three that held approximately 67 oysters each (215 total) and one empty tray that acted as a lid. Shallower depths were chosen for the oysters to maximize their encounter with sea lice larvae and natural food sources.

Oyster Growth Measurements

Oysters were sampled for growth approximately every four months, starting in late July 2013 when they were deployed, and then in November 2013, March 2014, and August 2014 before they were removed from the site. Ten oysters were sampled from each depth x side treatment level (1, 3, and 6 m depths; sides A—E at the farm, and F being the reference site), giving a total *n* of 180. These live oysters were transported back to the laboratory for processing. Oyster size at each sampling point was assessed by whole wet weight, soft tissue wet and dry weight, soft tissue ash-free dry weight (AFDW), and by shell length, width, and height. Whole wet weight and soft tissue wet weight were measured. Soft tissue of each oyster was then placed into individual pre-ashed and pre-weighed aluminum weigh boats and dried to constant weight at 60˚C (this took approximately 7–9 d). Final dry weight was recorded and the dried oyster tissue was then ashed in a muffle furnace (Thermolyne 30400, Thermo Fisher Scientific, Waltham, MA) for 6 h at 500˚C. Ash-free dry weight (organic weight) was calculated by subtracting the ash weight from the soft tissue dry weight. Oyster shell length, width, and height (Galtsoff, 1964) were measured using a digital caliper (Absolute Digimatic, Mitutoyo, Kawasaki, Japan).

Larval Sea Lice

Monthly vertical plankton hauls were performed, in triplicate, within three experimental salmon cages (with oysters) and three control cages (without oysters) at the opposite end of the farm to compare planktonic larval (nauplii and copepodid) sea lice densities. Note that these monthly larval densities were previously part of the dataset presented in Chapter 2, though not separated by bivalve treatment. The plankton net was made of 150-µm mesh and was 0.5-m in diameter and 1.5-m long (Dynamic Aqua-Supply Ltd., Surrey, BC).

Plankton samples were collected within an hour of high tide, typically over 2–3 d each sampling month. To sample, the plankton net with an attached flow meter (General Oceanics, Miami, FL) was lowered slowly to the bottom of the pen, approximately 18 m. The net was then vertically hauled up to the surface of the pen at approximately 0.5 $m \cdot s^{-1}$. Once at the surface, the flow meter reading was recorded and the net was immediately rinsed from the outside using seawater in a manual pump garden sprayer. The sample was concentrated into the net's cod end and rinsed into a bottle(s) containing 10% formaldehyde, buffered using 1-µm filtered seawater, and brought back to the Pacific Biological Station (PBS) for analysis.

Preserved samples were carefully rinsed with tap water in a 150-µm sieve to remove formaldehyde. Custom plankton raceways were used to examine the samples for sea lice; all samples were examined in full under a dissecting microscope (SMZ1000, Nikon, Tokyo, Japan). All sea lice were identified and counted. Sea lice nauplii were not identified to stage (1 or 2) or to species, but were likely *L. salmonis* or *C. clemensi* as later stages of both species were present at the farm throughout the experiment. Once

complete (fully examined for sea lice), the samples were rinsed into 70% ethanol for long-term storage. All identified sea lice were removed and stored in 70% ethanol, separate from the rest of the sample. Larval densities were calculated as the number of larvae (nauplii and copepodids) in the sample divided by the volume of water filtered, calculated according to the flow meter manufacturer's instructions.

The intention was to also perform monthly attached sea lice counts in the same three bivalve and three control cages used for the plankton hauls. It was decided, however, that this repeated sampling would have caused unwarranted stress on the fish in those pens and counts were generally only performed on three random cages per month, in accordance with federal regulations (Saksida et al., 2011b).

Ingested Sea Lice – Laboratory Experiment

A preliminary laboratory experiment was conducted to assess how long *L. salmonis* DNA remains detectable in the digestive tract of Pacific oysters, following their ingestion of *L. salmonis* copepodids. Pacific oysters (shell height: 85.8±1.4 mm, mean±SE) used in the experiment were brought to PBS from the salmon farm in late September 2013 and held in a seawater tank with flow-through, 1-µm filtered and UV treated seawater at 10˚C (±0.5˚C) at a salinity 29—30 PSU. Oysters were fed cultured microalgae (*Tisochrysis lutea*) weekly.

Gravid *L. salmonis* females were collected from a commercial salmon farm in October 2013 during harvest and transported to PBS. Egg strings were collected from the females and examined under a dissecting microscope. Viable eggs (*i.e.* neat, clean stacks) were kept and sorted into containers of 1-µm filtered and UV treated seawater (salinity

29–30 PSU) according to their approximate point of development. Each container was equipped with two air stones and held at 6.5±0.5˚C by a water bath. In November 2013, enough larvae had developed into copepodids and the experiment was commenced.

Oysters were starved for one week leading up to the experiment. Randomly selected oysters were measured (whole wet weight and shell length, width, and height) and placed individually into static experimental containers (10 cm diameter, 18 cm height cylinders), each containing a gently bubbling air stone, 24 h prior to the experiment. These containers were held in a larger flow-through seawater tank filled with 1-µm filtered seawater. Water temperature leading up to and during the experiment was 10.0±0.1˚C and salinity during the experiment was 29.0—29.5 PSU. Oysters exposed to sea lice and algae (as described in subsequent paragraphs) were sampled at 0, 1.5, 3, 6, 12, or 24 hours post-feed (hpf). Two controls were used: one with an oyster but no feed (algae or sea lice) and one with feed but no oyster. All of the time points and controls were run in triplicate with replicate containers being placed in the tanks randomly.

Half an hour before the experiment, 250 mL of *T. lutea* (180,000±60,000 cells·mL^{-1}) were added to each experimental container (final concentration approximately 45,000 cells·mL^{-1}), except for the three no-feed controls, to stimulate filter feeding. Finally, 100 copepodids were added to each container (except for the three no-feed controls) and the volume of all containers was topped up to 1 L with 1-µm filtered seawater as necessary. Oysters were exposed to copepodids for 1 h, after which bivalves were rinsed over their container and those not being sampled at this 0 h post-feed stage were transferred into an adjacent, identically-sized container of 1 L filtered seawater where they were held without food until sampling at 1, 3, 6, 12, or 24 h later. Water from

the first container was sieved and preserved in 70% ethanol to examine later under a dissecting microscope for remaining copepodids. The same was done for water from the second container (not used for oysters sampled 0 hpf). Oyster digestive tissue was chopped into small pieces using a sterile scalpel and tissue wet weight was recorded before preservation in 95% ethanol.

To analyze oyster tissue for sea louse DNA, samples were first centrifuged at 3000 g for 10 min so that ethanol could be decanted with minimal tissue loss. After decanting, oyster tissue was homogenized using a Polytron (Brinkmann Instruments, Mississauga, ON) and a portion (approximately 0.75 mL) was subsampled and frozen at -20°C. During the homogenization process a tissue positive (TP) control was created, which was one preserved oyster spiked with 10 *L. salmonis* copepodids before homogenization. Two negative tissue controls (oyster with no sea lice added) were also homogenized as an additional check for cross-contamination. Total DNA was extracted from 20—25 mg (wet weight) of each sample using the DNeasy® Blood & Tissue Kit, spin-column protocol (Qiagen, Hilden, Germany) and the methods provided by the kit. All samples were lysed overnight in Buffers ATL and Proteinase-K (Qiagen). After extraction, DNA concentration and purity were assessed using a NanoDrop™ (Thermo Fisher Scientific). Conventional PCR was used to amplify the *L. salmonis* mitochondrial cytochrome oxidase I (mtCOI) gene (102 bp) using the *L. salmonis* primers developed by McBeath et al. (2006). Previously tested *L. salmonis* copepodid DNA was used as a PCR positive control; water served as a negative PCR control. The PCR reaction volume was 25 μL total, 5 μL template and the remaining 20 μL a mastermix containing (final concentrations) 1x PCR reaction buffer (Invitrogen, Carlsbad, CA), 1.5 mM magnesium

chloride (Invitrogen), 0.2 mM dNTPs, 0.5 uM forward primer and 0.5 mM reverse primer (Eurofins Genomics, Huntsville, AL), and 0.025 $U \cdot uL^{-1}$ Platinum® *Taq* DNA polymerase (ThermoFisher Scientific). A Dyad® Peltier thermal cycler (Bio-Rad, Hercules, CA) was programmed to 95˚C for 3 min, followed by 45 cycles of 95˚C for 15 seconds (denaturation) and 60˚C for 1 min (annealing), finishing with a final extension at 72˚C for 10 min. PCR products were run through a 3% agarose gel for 40 min at 70 volts with a Low DNA Mass Ladder (Invitrogen). Gels were imaged using a Gel Doc-It™ (UVP, Upland, CA) imaging system and assessed for presence/absence of the target *L. salmonis* gene. Sensitivity of the PCR assay was determined by diluting both pure *L. salmonis* DNA and by diluting *L. salmonis* copepodids into oyster tissue; five *L. salmonis* copepodids were added to each of 2, 6, and 10 g (wet weight) Pacific oyster tissue, in triplicate.

Ingested Sea Lice – Field Experiment

In November 2013, January 2014, and February 2014, oysters were preserved in the field individually in containers of 95% ethanol. Nine oysters (three from each of three randomly selected stacks) were sampled for each of the six side and three depth treatment combinations (total n=162). Excess gonad (on large oysters) and adductor muscles were not preserved. Samples from January 2014, the month in which sea lice larval densities were highest that winter, were analyzed for the presence of *L. salmonis* DNA. Samples were processed in the same manner as those from the laboratory experiment, except DNA was extracted using the DNeasy® 96 Blood & Tissue Kit (Qiagen) and gels were run at 60 volts for 60 min.

Statistical Analyses

Statistical analyses were performed using SPSS® 21 (SAS® Institute Inc., Cary, NC). Oyster growth was analyzed using a general linear model (GLM) with time and depth as categorical, fixed factors, side as a blocked factor, and average initial size (within each depth/side combination) as a continuous covariate. Side was considered an arbitrarily-defined block factor; oysters could have instead been blocked by fish cage, cardinal direction, *etc*. As such, block interactions were not considered in the GLM (see Model 2, Newman et al., 1997). Initial size was added as a covariate to account for any differences in how oysters were randomly assigned to the sides and depths. Interactions between this continuous covariate and the categorical factors (time, depth, side) were not included in the model (Engqvist, 2005). The effect of side and depth on final oyster size measurements (in August, 2014) was assessed using a 2-way ANOVA. Initial size was not included as a covariate due to its lack of statistical significance in the growth GLM.

Monthly average sea lice larval densities were calculated by first averaging the triplicate from each pen, creating one independent point, then averaging these mean values across experimental (bivalve, n=3) and control (non-bivalve, n=3) cages. The effect of time and treatment on sea lice larval density was assessed using a two-way ANOVA.

Levene's tests were performed on the datasets to assess homogeneity of variance. Normality of the datasets was assessed visually using Q-Q plots and tested using a Kolmogorov-Smirnov (K-S) test. Due to deviations from normality and/or homogeneity of variance, all oyster size variables were transformed by taking the natural logarithm

(ln). All ln-transformed oyster size variables passed assumptions of normality (visually with Q-Q plots, and K-S test $p>0.05$) and homogeneity of variance (Levene's test, $p>0.05$) with the exception of soft tissue WW and soft tissue AFDW which did not pass the homogeneity of variance assumption ($p=0.034$ and $p<0.001$, respectively). Linear models were performed for these two variables despite the variance assumption failure. SNK post-hoc tests were performed on all significant ANOVAs. Statistical significance was reported when $p<0.05$.

Results

Bivalve Growth

In the 13-month field trial oysters grew larger over time in all variables measured, with the largest percentage change observed in the soft tissue AFDW (organic weight) (150% increase, Table 5). Time and side factors had a significant effect on all growth variables examined while the effect of depth was significant for all variables except for shell height ($p=0.611$) and shell length ($p=0.090$) (Table 6). No significant time and depth interaction was identified for any of the variables. The covariate, initial size, had no significant effect on the models, with the exception of whole wet weight ($p=0.037$) and was omitted from the following analyses

The effect of time was examined across all depths and sides using a 1-way ANOVA with SNK post-hoc tests (Figure 10). All size variables significantly increased over time, though four of the seven variables did not increase significantly between November 2013 and March 2014. The final measurements, taken in August 2014, were significantly larger than previous measurements for all of the variables (Figure 10). The

effect of depth (across all times and sides) was also assessed using a 1-way ANOVA and SNK post-hoc tests (Figure 11). Oysters at 1 and 3 m were significantly larger than oysters at 6 m for all variables except shell height and shell length (Figure 11).

Final measurements (in August 2014) for each oyster growth variable at each side/depth treatment are presented in Figure 12 through Figure 18. Significant differences among depths and/or sides were detected in all growth variables at the final sampling point except shell height (Figure 16). Oysters on sides A—C were generally larger than oysters on D—F, F being the control site (Figure 9). Oysters at 1 and/or 3 m were significantly larger than oysters at 6 m for all variables except for shell height (Fig. 12—18). There was no significant side x depth interaction for whole wet weight and all shell measurements, whereas soft tissue wet weight, dry weight, and AFDW showed a significant side x depth interaction and as such all side/depth combinations (n=18) were compared separately (Table 7).

Larval Sea Lice

ANOVA results revealed that there was no significant effect of time (p=0.522), bivalve treatment (p=0.167), or interaction between the two variables (p=0.809) on sea lice larval densities (Table 8). Although mean larval densities were almost always lower in the experimental cages with oysters than the control cages without them (Figure 19), the data were highly variable and no significant treatment effect was detected. 1-way ANOVA (treatment, across all times) was not significant (p=0.149).

Ingested Sea Lice – Laboratory Experiment

Preliminary testing to establish sensitivity of the PCR assay determined the limit of detection for pure *L. salmonis* DNA to be $1.2x10^{-3}$ ng·µL^{-1}. Sea lice DNA was detectable in all three replicates in the 2- and 6-g spiked oyster samples, and in two of the three 10-g samples. Finally the duration of detectability of louse DNA in the digestive tracts of oysters, post- 1 h feed treatment, was determined. *Lepeophtheirus salmonis* DNA was detectable in at least one oyster from each time point triplicate (Table 9). Sea lice DNA was not detected in any of the control oysters (Table 9, Figure 20).

Ingested Sea Lice – Field Experiment

There was no evidence of *L. salmonis* DNA in oysters sampled at the salmon farm in January 2014, when observed larval sea lice densities were highest (Figure 21). Samples that appeared positive for *L. salmonis* DNA (Figure 22) were extracted adjacent to tissue positive controls and were most likely contaminated during the extraction process. These samples were re-extracted and re-amplified and were negative for *L. salmonis* DNA.

Discussion

Bivalve Growth

Over 13 months, Pacific oysters cultured at a commercial Atlantic salmon farm in BC grew significantly larger in all of the seven growth variables measured: whole wet weight; soft tissue wet, dry, and AFDW; and shell height, length, and width. Oyster

growth increased over the first four months, from August until November 2013, and all variables were significantly increased in the last four months, between March and August 2014 (Figure 10). However, from November 2013 through March 2014, soft tissue dry weight, soft tissue AFDW, shell height, and shell width showed no significant increase in size. Consistent with this stagnated growth, a multi-year study by Harrison et al. (1983) reported plankton density food availability values near zero (<1 $mg \cdot m^{-3}$) in the region from November through February. Spring plankton blooms in March or April then cause plankton densities to increase upwards of 15 $mg \cdot m^{-3}$ (Yin et al., 1996). In addition to less plankton (*i.e.* food) being available in the water column during the winter, oysters may have been utilizing energy reserves towards metabolism and gametogenesis during that time (Stirling and Okumuş, 1995).

Culture depth is an important consideration for shellfish aquaculture due to its site-specific influence on water temperature and salinity, nutrient availability, predation, and other parameters that may impact shellfish survival and growth. All oysters in this study were positioned in the upper portion of the water column to maximize their encounters with phytoplankton and zooplankton, including sea lice larvae (Costelloe et al., 1996; McKibben and Hay, 2004; Norði et al., 2015; Penston et al., 2011, 2004). Despite this, oysters were significantly larger when grown in trays held at 1 and/or 3 m depth as opposed to 6 m depth (Figure 11). Ngo et al. (2006) reported a similar depth effect for *C. gigas* cultured on suspended long-lines. Oysters were significantly larger and had a greater reproductive output when positioned in the top 2 m of the long-line compared to the bottom 3—5 m, which the authors attributed to warmer water temperature and increased phytoplankton abundance (Ngo et al., 2006). An important

drawback that was encountered while culturing oysters near the surface was that the trays and ropes quickly became covered in biofouling (predominantly mussels and barnacles, and kelp in the spring) and frequent pressure washing was required to remove this biofouling and ensure water flow to the oysters.

Oyster growth was compared between the farm and reference site by considering the reference site as its own "side". Side had a significant ($p<0.001$) effect on linear models of all variables over the whole experiment. Comparing only the final measurements (taken in August 2014), side had a significant effect on all variables except shell height – the largest shell measurement of an oyster. This suggests that while oyster growth was improved on certain sides of the salmon cages (A—C were most often the sides significantly larger than F, the control site (Figure 9)), as seen in previous studies with bivalves and salmon (Jiang et al., 2012; Jones and Iwama, 1991; Macdonald et al., 2011; Stirling and Okumuş, 1995), nutrient delivery and/or other conditions differed at a finer scale than simply "farm" versus "reference site". Karayücel and Karayücel (2000) reported a similar position effect on mussels (*Mytilus edulis*) cultured in lantern nets suspended from a raft at 2 and 6 m depths. Mussels on the inflow end of the raft were exposed to more particulate organic matter (POM) and grew significantly larger than mussels on the outflow (Karayucel and Karayucel, 2000). Unlike the present study, though, no depth effect on bivalve growth (or POM) was detected (Karayucel and Karayucel, 2000).

Nutrient flows around aquaculture sites (dictated by local current patterns, winds, infrastructure, *etc.*) is an important area of IMTA research with obvious implications for the strategic placement of extractive species such as bivalves. Various techniques have

been used to quantify and describe nutrient flows including mathematical models (*e.g.* Cranford et al., 2013), laboratory simulations (*e.g.* Turner et al., 2015), and direct sampling of the water column (*e.g.* Brager et al., 2015; Lander et al., 2013). Measuring bivalve growth over time as done in the present study is an indirect measure of nutrient presence and can help unveil cumulative, site-specific spatial and temporal characteristics that affect the growth of this organic extractive IMTA component. Water current was not monitored as part of this study but was measured at the site over a 1-month period in 2011, prior to the farm's construction and the commencement of this experiment (data not shown). No dominant current flow direction was identified at 15 m, the most shallow and relevant current meter results, that would explain differences in bivalve growth. However, current patterns may exist closer to the surface and/or due to the addition of farm infrastructure and fish, meaning flow cannot be discounted as a contributing factor to the significant effect of cage side on bivalve growth. Cage infrastructure (metal walkways on floats, mesh netting, *etc*.) in particular has been shown to interrupt current flow, creating eddies and turbulence, as well as areas of very low current velocity within or close to the cages (Turner et al., 2014). Slower current speeds give bivalves a greater opportunity to filter the water, thus enhancing nutrient capture efficiency (Cranford et al., 2013). Overall, the optimal placement of oysters (in terms of maximizing somatic growth) was on cage sides A, B, and C at 1 and 3 m.

Sea Lice Mitigation

With the exception of October 2013, mean sea lice larval density was lower, though not significantly, in bivalve cages compared to non-bivalve cages. The non-

significance of time, treatment, and their interaction on sea lice larval density was likely a result of large variation within the monthly sample data (Table 8, Figure 19). With the variation encountered, and using the experimental design described, a retrospective power analysis suggested a required sample size (of plankton hauls) in the hundreds per month to detect a 10–20% biological effect. Due to the dynamic nature of the plankton community and its ability to drift in and out of salmon net pens, presence of sea lice larvae in control/experimental pens does not equate to the lice originating from those pens. Attempts were made to control the sea lice larval "snapshots" provided by monthly plankton samples in order to tease out the effect of bivalves, such as the placement of control and experimental cages at opposite ends of the farm (as far away from each other as possible) and sampling the cages randomly at consistent tidal levels. Still, factors other than oysters (e.g. winds moving surface waters (Norði et al., 2015)) would have had an effect on observed densities in treatment and control cages.

Larval densities were highest in January 2014 which is why oyster digestive tract samples preserved this month were analyzed for sea louse DNA presence. Prior to the analysis, a preliminary laboratory experiment suggested that DNA remained detectable up to 24 hours post feeding and indicated reduced likelihood of detection over time, which was anticipated. The negative oyster from 12 hpf had 84 of 100 *L. salmonis* copepodids remaining in its experimental containers, the highest of the 12 hpf triplicate, and thus presumably had ingested the fewest copepodids for that treatment. However, of the three oysters sampled at 24 h, the only oyster to test positive for *L. salmonis* DNA had the *most* copepodids remaining in its experimental seawater (75, compared to 38 and 28 remaining in the DNA-negative samples for 24 hpf). Since bivalve filter-feeding was

not directly quantified, inferring louse ingestion from the fraction of remaining copepodids has important limitations. It is difficult to say conclusively whether sea lice DNA was not detected simply because the larvae were not ingested, or because digestion rendered the DNA signal undetectable. Presumably, the anomalous DNA-negative sample from 0 hpf can be explained by the former, while the two DNA-negative oysters sampled at 24 hpf likely did ingest some portion of the 62 or 72 copepodids "missing" from their respective containers' seawater. 100, 98, and 93 copepodids were recovered in seawater from the no-oyster control containers, suggesting both that the initial larvae counts were fairly accurate and that copepodid loss in the experimental containers was minimal.

Cross-contamination of louse DNA when homogenizing oyster samples was a concern and therefore a 10 copepodid-spiked oyster sample was processed alongside samples from the laboratory feeding experiment. This spiked sample was positive for louse DNA presence while the two negative tissue samples processed afterwards (in the same manner as all experimental samples) were negative for *L. salmonis* DNA, indicating cross-contamination was not an issue during this step of the analysis. Oyster samples from the salmon farm were later homogenized using the same methods.

Lepeophtheirus salmonis DNA was not detected in field oyster samples, with the exception of a few false positives. The PCR technique was able to detect low quantities of sea louse DNA in the laboratory (*e.g.* 5 copepodids diluted in 10 g wet weight of oyster tissue). However, sea lice larvae were present in farm water samples in very low densities (usually less than 1 $ind \cdot m^{-3}$) even during peak infection periods, meaning oysters would have to filter huge amounts of water to capture detectable quantities of lice

DNA and significantly reduce larval lice numbers. Furthermore, the PCR was specific for *L. salmonis* when planktonic larvae at the farm may have also been included *Caligus* species, making the density of larvae screened for potentially even lower. Preventative sea louse mitigation strategies targeting the planktonic phase of the louse life cycle would therefore benefit from concentrating the larvae, for example using artificial light (Flamarique et al., 2009) or current (Samsing et al., 2015). The feasibility of concentrating and capturing larvae (which would likely result in the concentration of other zooplankton and possibly phytoplankton) is aided by the fact that sea lice larval densities are highest during the winter when surrounding seston in the water column is relatively low.

Overall, with the methods used in this study, oysters had no significant effect on sea lice numbers at the farm, despite clear evidence of louse ingestion and digestion by bivalves in the laboratory (Bartsch et al., 2013; Molloy et al., 2011; Webb et al., 2013). It is possible that the number and/or positioning of oysters was simply insufficient to capture planktonic sea lice larvae or, if a portion of these larvae *were* ingested, they went undetected in the samples collected. Larval densities inside of the fish cages were lower than expected and sometimes varied greatly even within triplicate samples from the same pen. An alternate, perhaps more effective experimental design (which was not feasible in this study) would be to have multiple horizontal "layers" of suspended bivalves, spread out vertically in the water column (*e.g.* from 1 m down to 6 m). Bivalves would ideally be positioned in between the net pens in addition to the outer cage sides. A major drawback of this design, and of culturing shellfish in close proximity to fish cages in general, is the possible inhibition of water flow and thus oxygen to the fish cages – one of

many considerations for further development of practical and profitable finfish-bivalve IMTA.

Conclusions

The growth and sea lice mitigation ability of Pacific oysters at a commercial salmon farm was assessed. Pacific oysters are a dominant shellfish aquaculture crop in BC, but are typically cultured south of the study location in warmer, more sheltered waters. Further development of commercial-scale IMTA will likely necessitate growing species in novel locations and/or with novel infrastructure, where growth has not yet been measured or optimized. Oyster shell size and tissue weight increased significantly over the course of this experiment, the extent of which was dependent on both depth and location (side of the farm or reference site). Oysters in trays deployed at 1 and 3 m depths were often significantly larger than those at 6 m, suggesting that the optimal capture of nutrients from the natural seston and/or farm waste particulates occurred near the surface. The significant side effect emphasizes the importance of extractive species' positioning at IMTA sites. If side were not accounted for as a blocking factor, the study would have likely concluded there were no significant differences in oyster growth between the farm and reference site. Instead, oysters at the farm *were* significantly larger than those at the reference site in several of the sides/variables. Oysters did not appear to significantly reduce sea lice larval densities or ingest them, at least in ways detectable by our methods. Larval densities varied substantially among samples and this within-treatment variation hindered our ability to detect either a time or treatment effect. Oysters preserved in January 2014 showed no evidence of *L. salmonis* ingestion (DNA); either no sea lice

were present in the digestive tracts or they were present at levels below the PCR detection level. Regardless, the low densities of sea lice larvae observed inside of the fish cages poses a significant challenge for mitigation strategies targeting this phase of the life cycle. In the future, sea louse mitigation by bivalves will require their dense, strategic placement and concentrating the larvae using light or other means would be beneficial.

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Tables

Table 1: Monthly sea lice larval densities at the farm, averaged from six net pens, and at the reference site.

Sampling date	Farm larval density±SE (m^{-3})	Reference site larval density (m^{-3})
Dec. 2012	0.21 ± 0.17	-
Jan. 2013	1.28 ± 0.62	-
Feb. 2013	0.71 ± 0.29	-
Sep. 2013	0.00 ± 0.00	-
Oct. 2013	0.71 ± 0.12	-
Nov. 2013	0.64 ± 0.20	-
Dec. 2013	0.52 ± 0.19	-
Jan. 2014	0.96 ± 0.25	0.83$^{\psi}$
Feb. 2014	0.50 ± 0.15	0.66$^{\psi}$
Mar. 2014	0.39 ± 0.17	0.43$^{\psi}$

ψ indicates that no SE was calculated as only one sample was collected.
– indicates not sampled.

Table 2: Sea lice stages found in plankton tows from December 2012 to February 2013 and September 2013 to March 2014 at the farm and from January 2014 to March 2014 at the reference site.

	Number found, percentage of total		
Louse stage and species	**Dec. 2012–Feb. 2013** Farm (54 samples)	**Sep. 2013–Mar. 2014** Farm (141 samples)	**Jan. 2014–Mar. 2014** Reference Site (9 samples)
Nauplius (any species)	68, 73.9%	204, 93.3%	17, 100%
Copepodid (any species)	7, 7.6%	10, 4.2%	
Chalimus *C. clemensi*	13, 14.1%	4, 1.7%	
Motile *L. salmonis*	2, 2.2%	0, 0%	
Motile *C. clemensi*	2, 2.2%	2, 0.8%	

Table 3: Observed and estimated average monthly densities of sea lice nauplii at the farm.

Date	Observed nauplius density (m^{-3}) ± SE	Estimated nauplius density (m^{-3})	Estimated nauplius density, using gravids only (m^{-3})	Adult female *L. salmonis* per fish ± SE	Gravid *L. salmonis* per fish ± SE	Average temperature 6 m (˚C)	Average salinity 5 m (PSU)	Estimated number of fish on-site
Dec. 2012	0.16 ± 0.16	12.21	2.54	0.20 ± 0.05	0.04 ± 0.02	7.3	32.9	700,000
Jan. 2013	1.21 ± 0.63	21.71	10.13	0.38 ± 0.07	0.18 ± 0.05	7.0	33.9	700,000
Feb. 2013	0.68 ± 0.29	8.84	7.86	0.15 ± 0.05	0.13 ± 0.05	7.1	34.0	700,000
Sep. 2013	0.00 ± 0.00	25.36	11.53	0.28 ± 0.08	0.13 ± 0.05	9.9	28.5	700,000
Oct. 2013	0.56 ± 0.09	ψ	ψ	ψ	ψ	9.1	31.2	700,000
Nov. 2013	0.62 ± 0.19	37.93	28.45	0.53 ± 0.10	0.40 ± 0.09	8.2	33.0	700,000
Dec. 2013	0.51 ± 0.18	39.36	14.89	0.62 ± 0.11	0.23 ± 0.06	7.6	33.0	700,000
Jan. 2014	0.95 ± 0.25	103.74	50.33	1.68 ± 0.18	0.82 ± 0.12	7.4	33.1	700,000
Feb. 2014	0.48 ± 0.16	98.56	54.99	1.73 ± 0.22	0.96 ± 0.14	6.9	32.7	700,000
Mar. 2014	0.39 ± 0.17	19.14	7.66	0.33 ± 0.07	0.13 ± 0.04	7.0	32.9	700,000

ψ indicates that no lice counts were performed this month; nauplius density could not be estimated.

Table 4: Monthly mean parasite abundance and species composition on farmed Atlantic salmon, averaged from two to six pens.

Year	Month	Mean parasite abundance (lice·$fish^{-1}$)	Percentage *L. salmonis*	Percentage *C. clemensi*
2012	Dec.	1.76	25.5	74.5
2013	Jan.	6.47	13.0	87.0
	Feb.	2.13	20.9	79.1
	Mar.	0.12	4.2	95.8
	Apr.	0.43	9.1	90.9
	May	0.08	3.3	96.7
	Jun.	0.07	1.7	98.3
	Jul.	0.15	12.5	87.5
	Aug.	0.07	5.0	95.0
	Sep.	0.63	38.7	61.3
	Oct.	ψ		
	Nov.	1.23	47.7	52.3
	Dec.	1.52	53.7	46.3
2014	Jan.	2.53	83.3	16.7
	Feb.	3.28	80.9	19.1
	Mar.	0.80	30.9	69.1

ψ indicates that no lice counts were performed this month.

Table 5: Initial and final oyster size, averaged over all treatments, and the percent increase in size.

Growth variable	August, 2013 Initial size ± SE	August, 2014 Final size ± SE	Percent increase
Whole WW	39.5 ± 1.3 g	71.0 ± 1.3 g	79.6%
Soft tissue WW	12.7 ± 0.4 g	22.6 ± 0.4 g	78.0%
Soft tissue DW	1.752 ± 0.068 g	3.758 ± 0.091 g	114.5%
Soft tissue AFDW	1.307 ± 0.077 g	3.262 ± 0.084 g	149.8%
Shell height	81.1 ± 0.9 mm	94.0 ± 0.8 mm	15.8%
Shell length	52.8 ± 0.7 mm	59.3 ± 0.6 mm	12.4%
Shell width	23.5 ± 0.4 mm	28.7 ± 0.5 mm	22.2%

WW=wet weight, DW=dry weight, AFDW=ash-free dry weight.

Table 6: ANOVA results of general linear models for the various oyster growth variables over the experimental period. Significant *p*-values are shown in bold.

Growth variable	Term	df	Sum of squares	Mean square	*F*-ratio	*p*-value
ln (Whole WW)	Time	3	23.934	7.978	135.559	**<0.001**
	Depth	2	1.718	0.859	14.598	**<0.001**
	Side	5	8.376	1.675	28.465	**<0.001**
	Covariate	1	0.257	0.257	4.364	**0.037**
	Time x Depth	6	0.354	0.059	1.003	0.423
	Error	607	35.724	0.059		
ln (Soft tissue WW)	Time	3	35.730	11.910	178.737	**<0.001**
	Depth	2	3.668	1.834	27.525	**<0.001**
	Side	5	7.793	1.559	23.390	**<0.001**
	Covariate	1	0.024	0.024	0.364	0.547
	Time x Depth	6	0.293	0.049	0.734	0.622
	Error	607	40.466	0.067		
ln (Soft tissue DW)	Time	3	46.129	15.376	164.982	**<0.001**
	Depth	2	8.317	4.159	44.621	**<0.001**
	Side	5	9.922	1.984	21.292	**<0.001**
	Covariate	1	0.136	0.136	0.1455	0.228
	Time x Depth	6	0.586	0.098	1.048	0.393
	Error	607	56.573	0.093		
ln (Soft tissue AFDW)	Time	3	64.818	21.606	177.538	**<0.001**
	Depth	2	10.230	5.115	42.028	**<0.001**
	Side	5	15.796	3.159	25.959	**<0.001**
	Covariate	1	0.215	0.215	0.765	0.185
	Time x Depth	6	0.802	0.134	1.099	0.362
	Error	565	68.760	0.122		
ln (Shell height)	Time	3	1.371	0.457	41.269	**<0.001**
	Depth	2	0.011	0.005	0.493	0.611
	Side	5	0.386	0.077	6.979	**<0.001**
	Covariate	1	0.006	0.006	0.522	0.470
	Time x Depth	6	0.056	0.009	0.841	0.539
	Error	609	6.746	0.011		
ln (Shell length)	Time	3	1.057	0.352	19.777	**<0.001**
	Depth	2	0.086	0.043	2.414	0.090
	Side	5	1.304	0.261	14.647	**<0.001**
	Covariate	1	0.056	0.056	3.120	0.078
	Time x Depth	6	0.022	0.004	0.203	0.976
	Error	609	10.847	0.018		
ln (Shell width)	Time	3	2.577	0.859	27.567	**<0.001**
	Depth	2	0.525	0.263	8.430	**<0.001**
	Side	5	1.827	0.365	11.726	**<0.001**
	Covariate	1	0.014	0.014	0.465	0.496
	Time x Depth	6	0.340	0.057	1.819	0.093
	Error	609	18.977	0.031		

WW=wet weight, DW=dry weight, AFDW=ash-free dry weight.

Table 7: ANOVA results for the various oyster growth variables at the end of the experiment. Significant *p*-values are shown in bold.

Variable	Term	df	Sum of squares	Mean square	*F*-ratio	*p*-value
ln (Whole WW)	Depth	2	1.074	0.537	11.48	<**0.001**
	Side	5	2.238	0.448	9.456	<**0.001**
	Depth x Side	10	0.755	0.076	1.596	0.112
	Error	161	7.619	0.047		
ln (Soft tissue WW)	Depth	2	2.118	1.059	20.632	<**0.001**
	Side	5	2.224	0.445	8.664	<**0.001**
	Depth x Side	10	1.185	0.119	2.309	**0.015**
	Error	161	8.264	0.051		
ln (Soft tissue DW)	Depth	2	3.099	1.549	18.906	<**0.001**
	Side	5	3.392	0.678	8.278	<**0.001**
	Depth x Side	10	2.111	0.211	2.576	**0.006**
	Error	161	13.193	0.082		
ln (Soft tissue AFDW)	Depth	2	3.924	1.647	17.213	<**0.001**
	Side	5	3.930	0.786	8.213	<**0.001**
	Depth x Side	10	2.335	0.234	2.440	**0.010**
	Error	161	15.407	0.096		
ln (Shell height)	Depth	2	0.030	0.015	1.171	0.313
	Side	5	0.110	0.022	1.696	0.138
	Depth x Side	10	0.133	0.013	1.028	0.422
	Error	161	2.083	0.013		
ln (Shell length)	Depth	2	0.044	0.022	1.177	0.311
	Side	5	0.272	0.054	2.931	**0.015**
	Depth x Side	10	0.326	0.033	1.755	0.073
	Error	161	2.986	0.019		
ln (Shell width)	Depth	2	0.383	0.192	5.362	**0.006**
	Side	5	0.615	0.123	3.443	**0.006**
	Depth x Side	10	0.663	0.066	1.855	0.055
	Error	161	5.756	0.036		

WW=wet weight, DW=dry weight, AFDW=ash-free dry weight.

Table 8: ANOVA results comparing sea lice larval densities inside of experimental (bivalve) and control (non-bivalve) fish cages over time.

Parameter	df	Sum of squares	Mean square	*F*-ratio	*p*-value
Time	5	1.058	0.212	0.859	0.522
Treatment	1	0.500	0.500	2.030	0.167
Time x Treatment	5	0.554	0.111	0.450	0.809
Error	24	5.914	0.246		

Table 9: Results of laboratory experiment feeding *L. salmonis* copepodids to Pacific oysters and sampling over 24 h.

Treatment (h post-feed)	Shell height (mm)	Whole wet weight (g)	Copepodids in 1st seawater (container with feed)	Copepodids in 2nd seawater (container post-feed)	Total copepodids remaining in seawater	Wet weight of preserved tissue (g)	Sea lice detected in PCR
0	88.64	61.5	71	N/A	71	4.7	No
0	89.14	61.7	44	N/A	44	3.4	Yes
0	87.4	65.3	55	N/A	55	5.0	Yes
1.5	94.35	47.6	50	7	57	4.0	Yes
1.5	90.54	43.6	56	7	63	3.0	Yes
1.5	85.39	54.2	82	1	83	3.9	Yes
3	81.72	37.7	17	12	29	2.9	Yes
3	88.99	63.0	68	0	68	6.9	Yes
3	81.31	65.2	56	4	60	6.9	Yes
6	77.71	44.1	34	0	34	2.4	Yes
6	85.06	55.9	92	1	93	4.4	Yes
6	87.96	61.0	83	11	94	4.8	Yes
12	80.17	42.6	35	3	38	4.2	Yes
12	73.38	57.7	80	4	84	3.6	No
12	96.54	55.3	37	30	67	4.8	Yes
24	90.95	58.5	72	3	75	4.2	Yes
24	92.23	55.0	37	1	38	3.9	No
24	94.16	65.5	28	0	28	3.9	No
0 no lice	77.51	48.2	0	N/A	0	3.1	No
0 no lice	82.35	64.3	0	N/A	0	5.3	No
0 no lice	75.71	49.6	0	N/A	0	5.4	No
0 no oyster	N/A	N/A	93	N/A	93	N/A	N/A
0 no oyster	N/A	N/A	98	N/A	98	N/A	N/A
0 no oyster	N/A	N/A	100	N/A	100	N/A	N/A

Figures

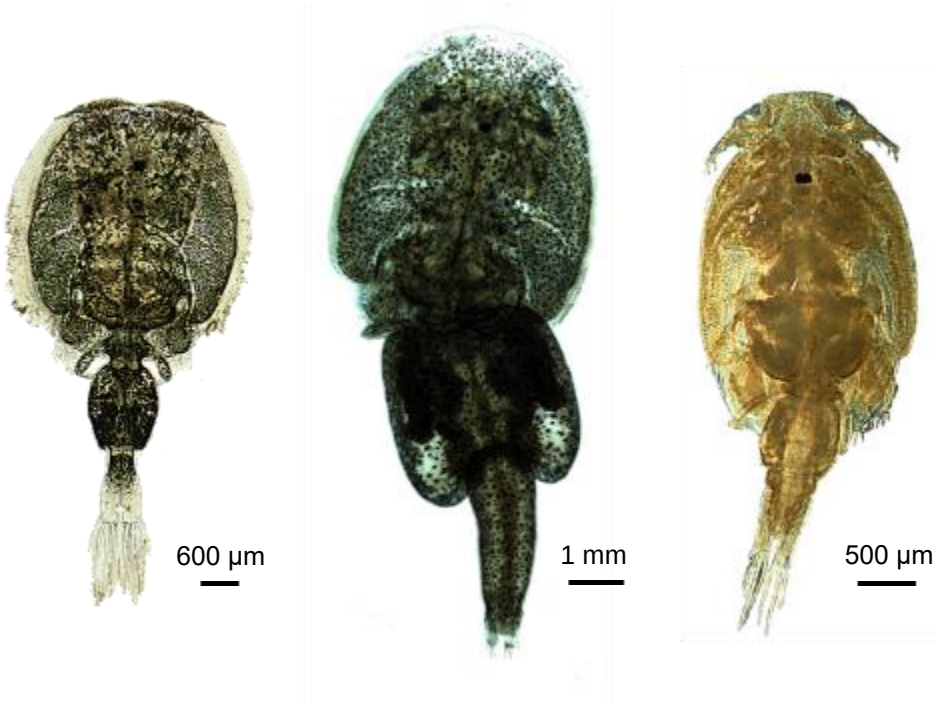

Figure 1: Sea lice commonly found on wild and farmed salmon in British Columbia. Left to right: *Lepeophtheirus salmonis* adult male, *L. salmonis* adult female, *Caligus clemensi* adult male.

Figure 2: *Lepeophtheirus salmonis* larvae. Left to right: nauplius 1, nauplius 2, copepodid.

Figure 3: Map showing study site location in the Pacific Ocean between Vancouver Island, British Columbia (BC), Canada and mainland BC.

Figure 4: Examples of sea lice larvae found in plankton samples. Left to right: sea louse nauplius in egg case, sea louse nauplius, *Lepeophtheirus salmonis* copepodid.

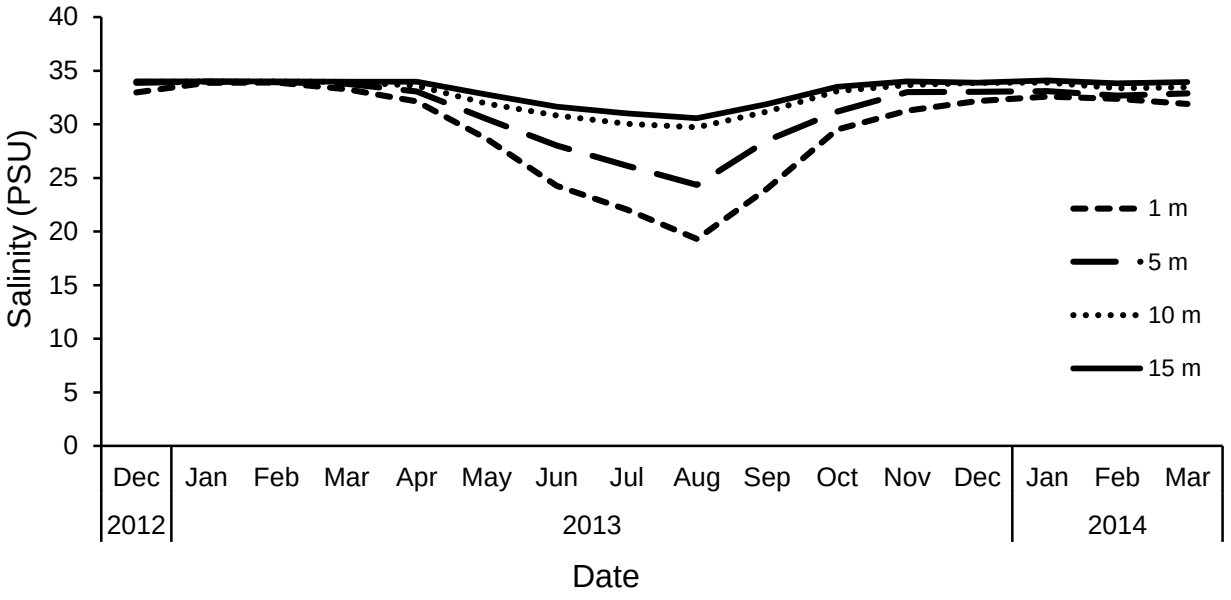

Figure 5: Water salinity profile at the salmon farm during the study period, December 2012 to March 2014.

Figure 6: Examples of chalimus stages found in plankton samples. Left to right: *Caligus clemensi* chalimus 1, *C. clemensi* chalimus 3, *C. clemensi* chalimus 4 female.

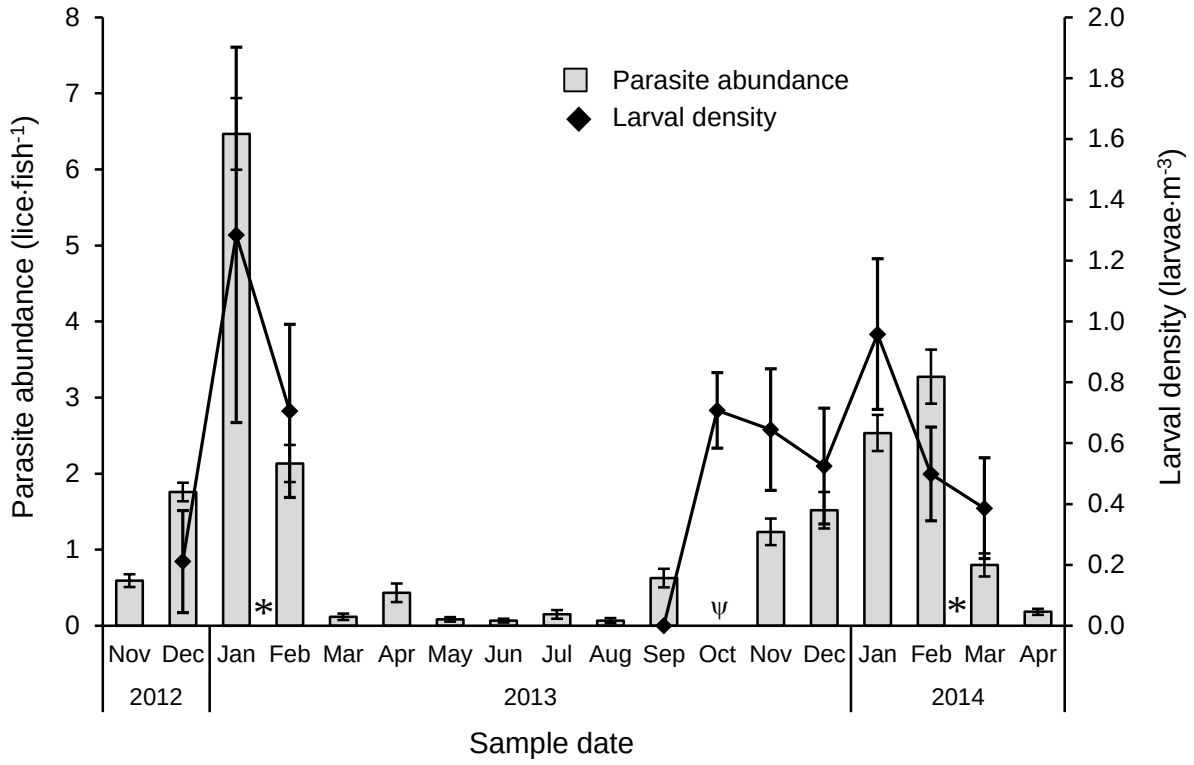

Figure 7: Average monthly sea lice larval density (*n*=6) and parasite abundance on fish (*n*≥60) at the salmon farm. Error bars are ±SE.
ψ indicates that no lice counts were performed in October 2013.
*** indicates timing of SLICE® treatment.**

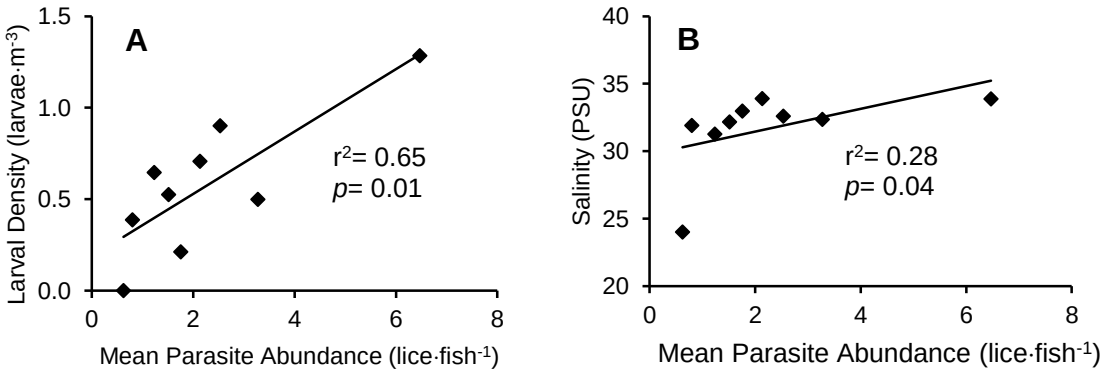

Figure 8: Correlation between mean parasite abundance and (A) larval density and (B) salinity at 1 m.

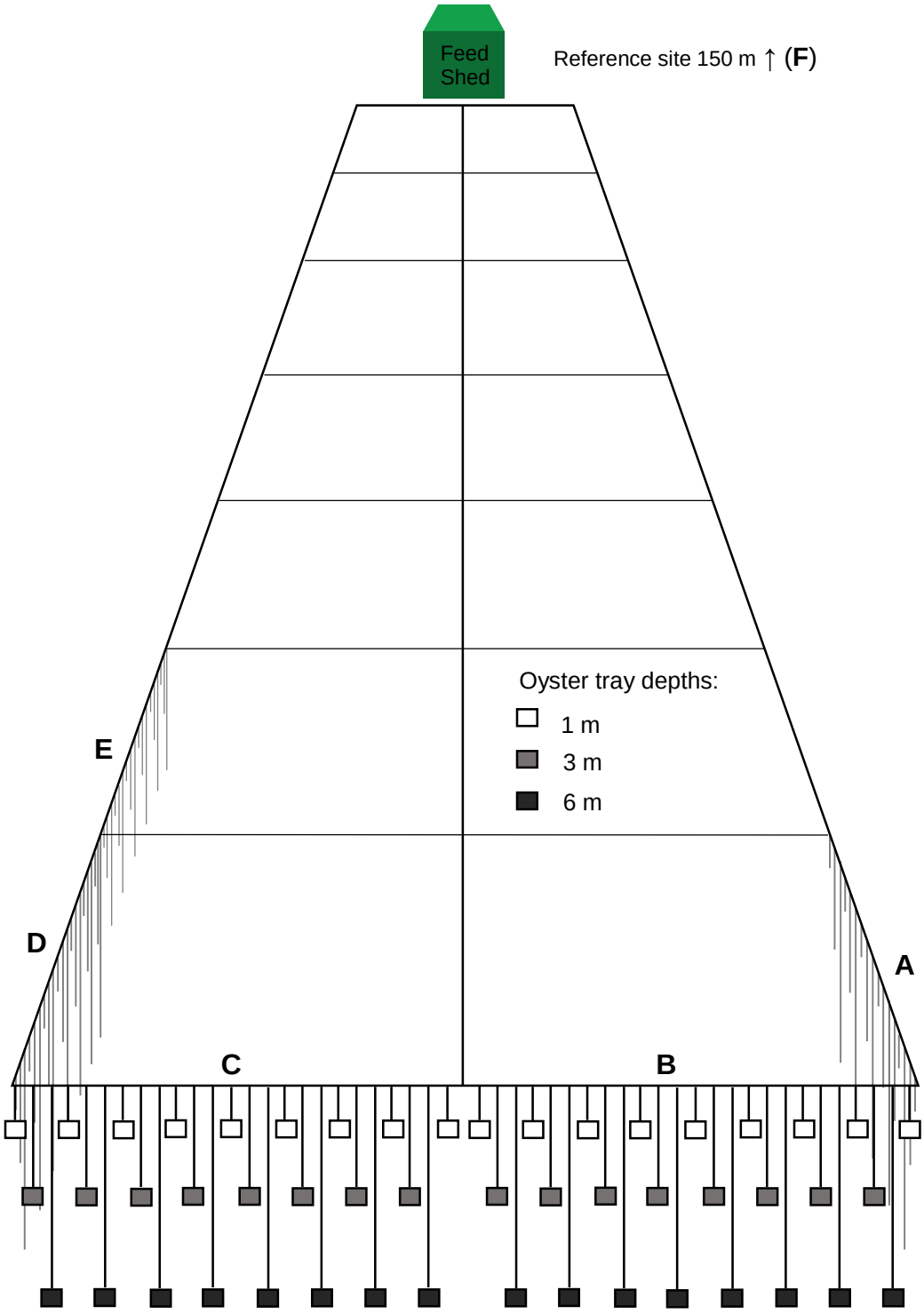

Figure 9: Schematic (not to scale) showing the salmon farm's 2x7 floating cage array and oyster tray arrangement at 1, 3, and 6 m depths on sides A–E; reference site (F) not shown. Each salmon pen is approximately 30 m x 30 m.

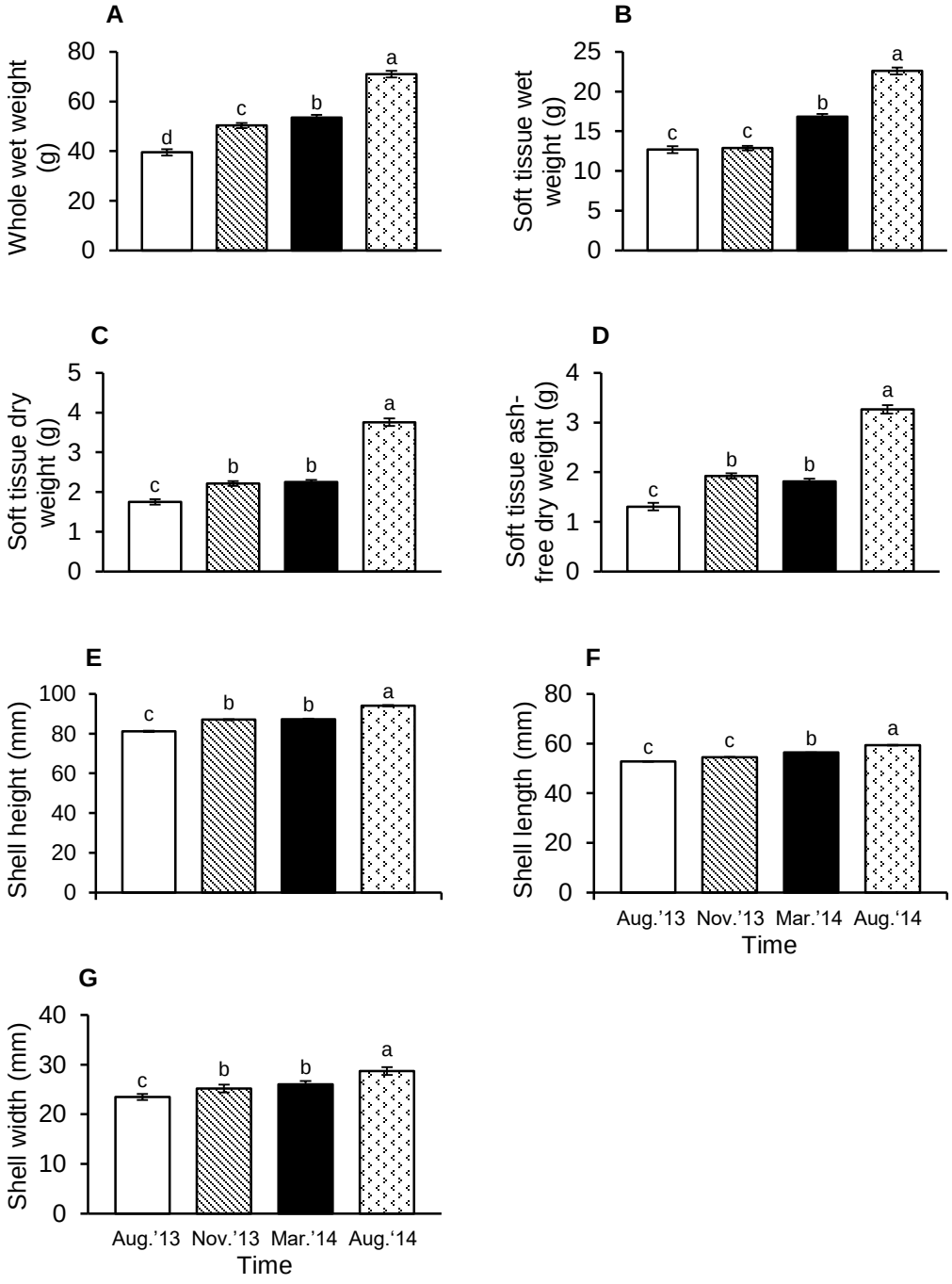

Figure 10: Mean oyster size at each time, across all depths and cage sides (A: whole wet weight, B: soft tissue wet weight, C: soft tissue dry weight, D: soft tissue ash-free dry weight, E: shell height, F: shell length, G: shell width). *n*=210 and error bars are SE. Different lowercase letters indicate significant ($p<0.05$) differences among times.

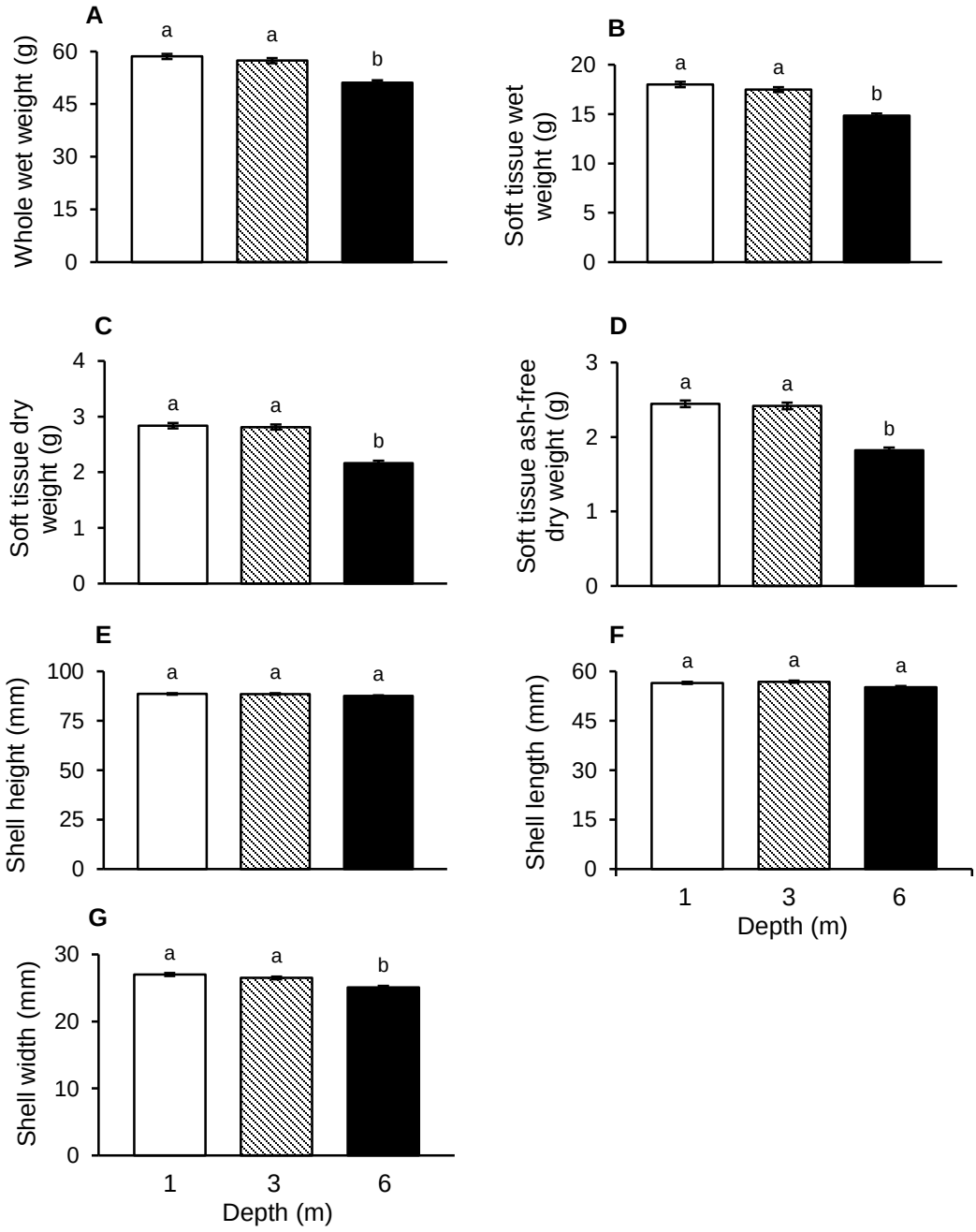

Figure 11: Mean oyster size at each depth, across all times and cage sides (A: whole wet weight, B: soft tissue wet weight, C: soft tissue dry weight, D: soft tissue ash-free dry weight, E: shell height, F: shell length, G: shell width). *n*=210 and error bars are SE. Different lowercase letters indicate significant ($p<0.05$) differences among depths.

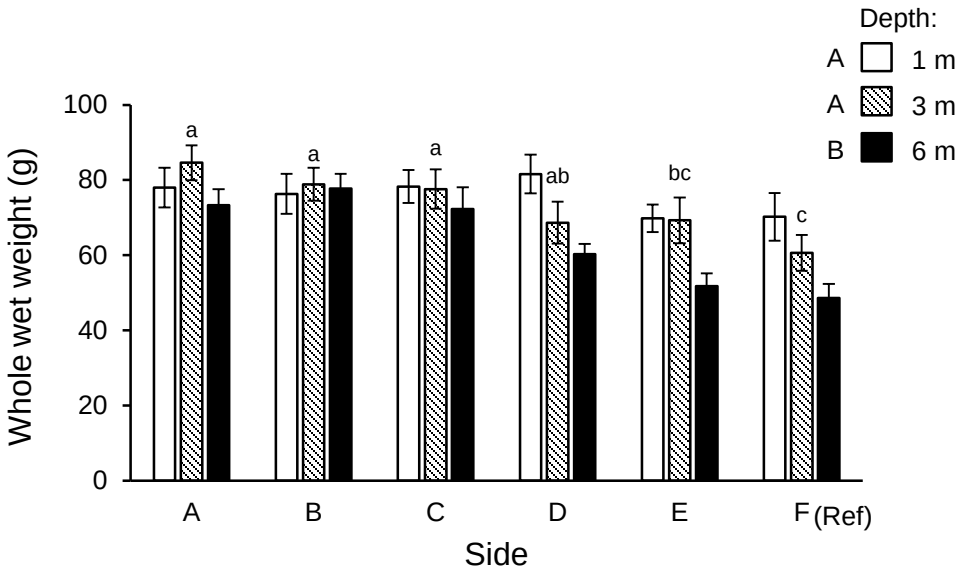

Figure 12: Mean whole wet weight of oysters in August 2014 (the final sampling point). *n*=10 and error bars are SE. Different lowercase and uppercase letters indicate significant (*p*<0.05) differences among cage sides and depths, respectively.

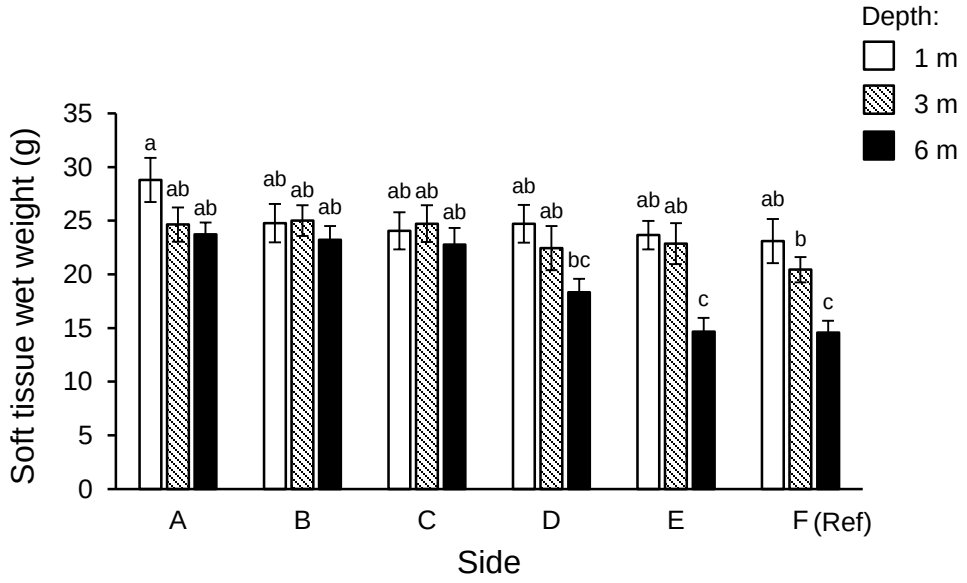

Figure 13: Soft tissue wet weight of oysters in August 2014 (the final sampling point). *n*=10 and error bars are SE. Different lowercase letters indicate significant (*p*<0.05) differences among all cage side and depth combinations.

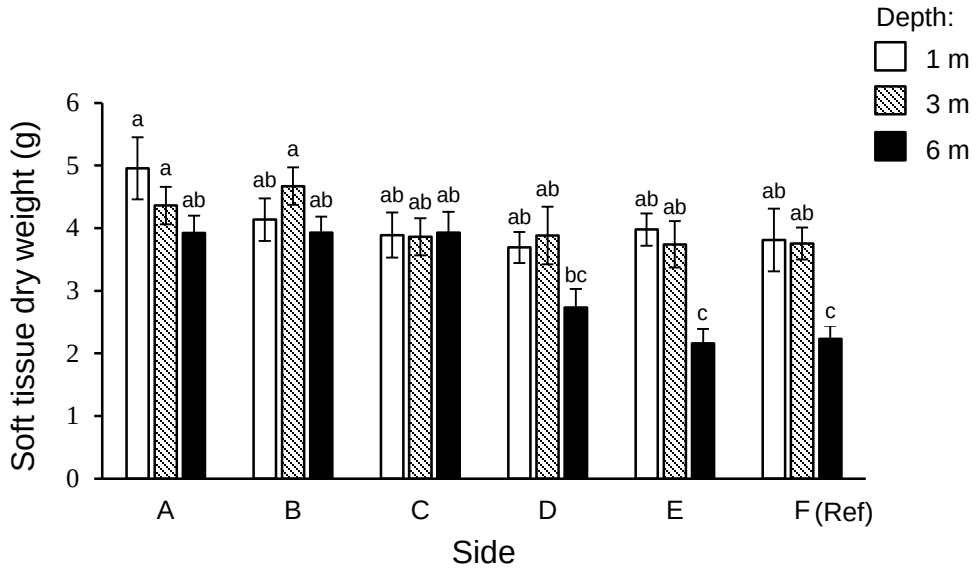

Figure 14: Soft tissue dry weight of oysters in August 2014 (the final sampling point). *n*=10 and error bars are SE. Different lowercase letters indicate significant (*p*<0.05) differences among all cage side and depth combinations.

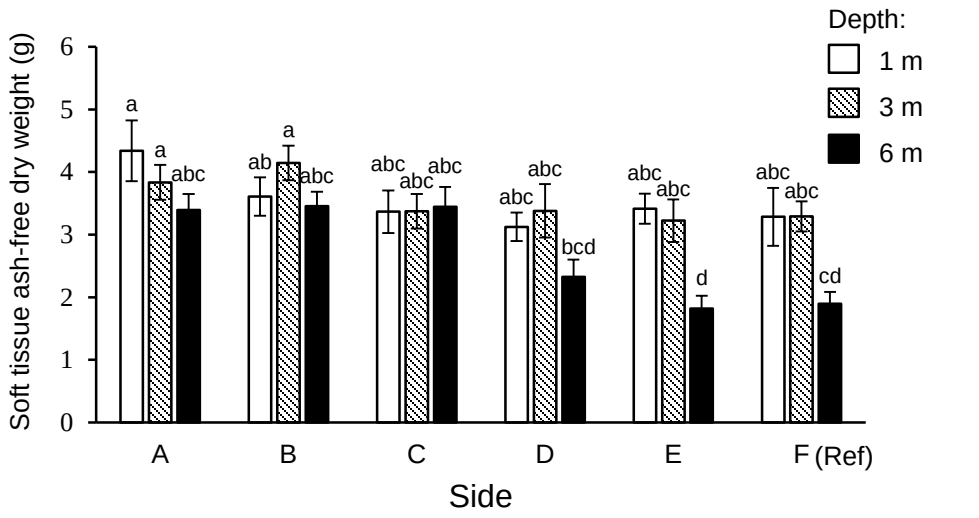

Figure 15: Soft tissue ash-free dry weight of oysters in August 2014 (the final sampling point). *n*=10 and error bars are SE. Different lowercase letters indicate significant (*p*<0.05) differences among all cage side and depth combinations.

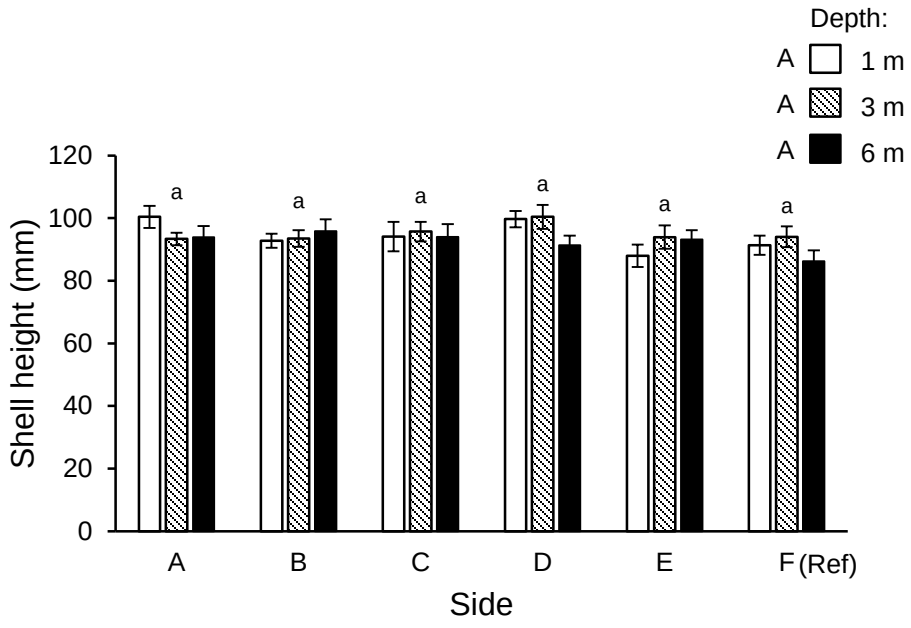

Figure 16: Shell height of oysters in August 2014 (the final sampling point). *n*=10 and error bars are SE. Different lowercase and uppercase letters indicate significant (*p*<0.05) differences among cage sides and depths, respectively.

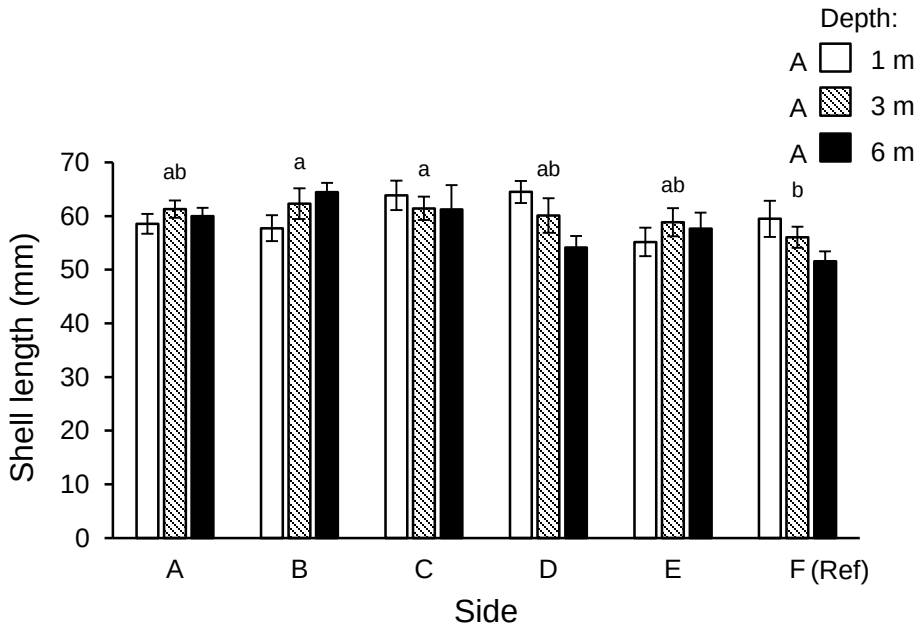

Figure 17: Shell length of oysters in August 2014 (the final sampling point). *n*=10 and error bars are SE. Different lowercase and uppercase letters indicate significant (*p*<0.05) differences among cage sides and depths, respectively.

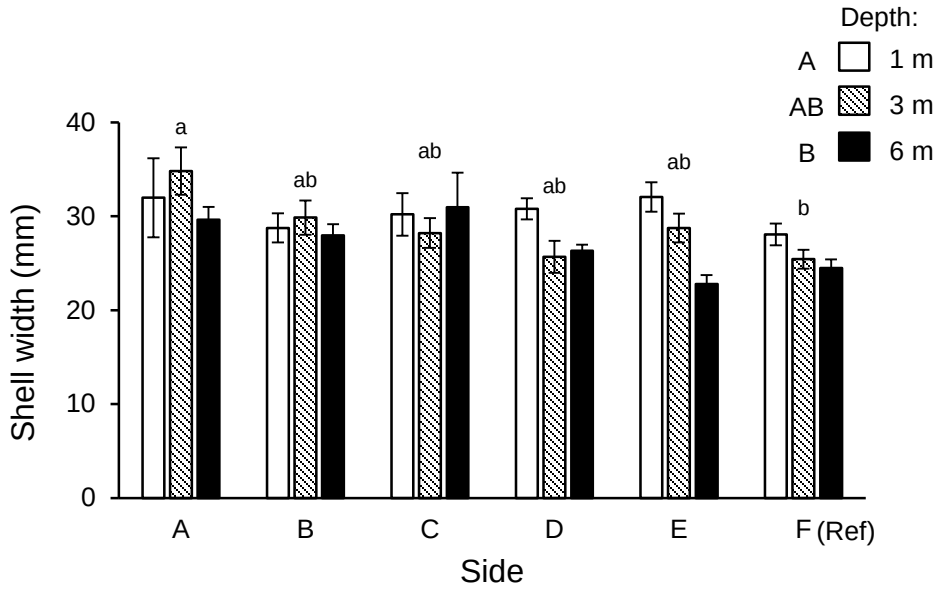

Figure 18: Shell width of oysters in August 2014 (the final sampling point). *n*=10 and error bars are SE. Different lowercase and uppercase letters indicate significant (*p*<0.05) differences among cage sides and depths, respectively.

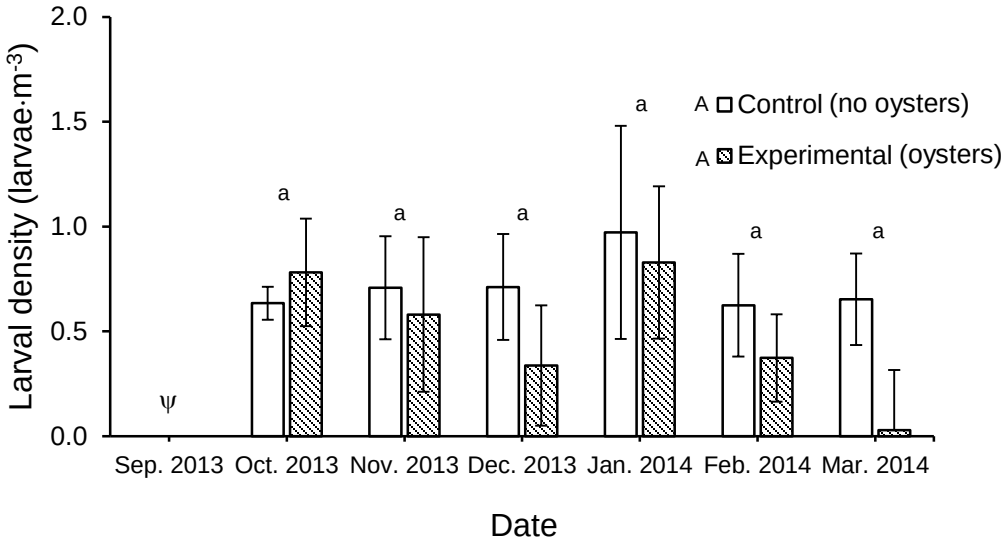

Figure 19: Monthly sea lice larval density (±SE) inside of control (bivalve, *n*=3) and experimental (non-bivalve, *n*=3) fish cages between September, 2013 and March, 2014. No significant effects of treatment (*p*=0.71) or time (*p*=0.44) were detected. ψ indicates that no sea lice larvae were found in September 2013 samples.

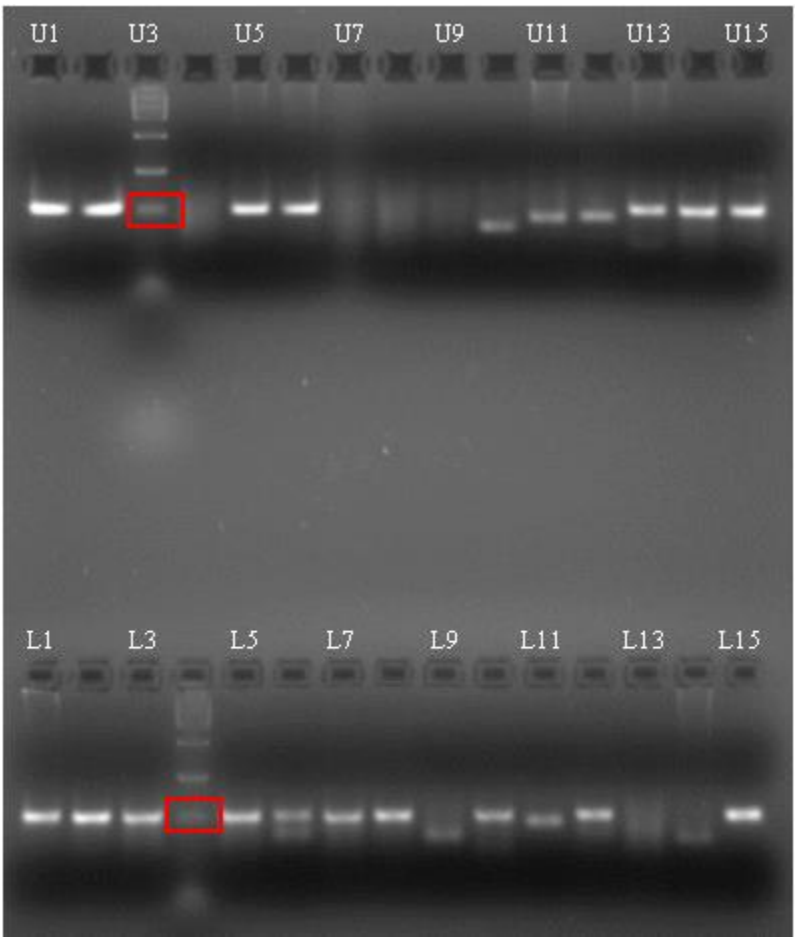

Figure 20: Agarose gel of laboratory sea lice feeding experiment samples. 100 bp bands of ladders are outlined in red boxes. The target *L. salmonis* gene was 102 bp. Lane composition: U1 tissue positive; U2 DNA positive; U3 ladder; U4-6, 0 h; U7-9, 0 h no lice; U10-11 tissue negative; U12 negative, U13-15, 1.5 h; L1-3, 3 h; L4 ladder; L5-7, 6 h; L8-10, 12 h; L11 negative; L12-14 24 h; L15 DNA positive.

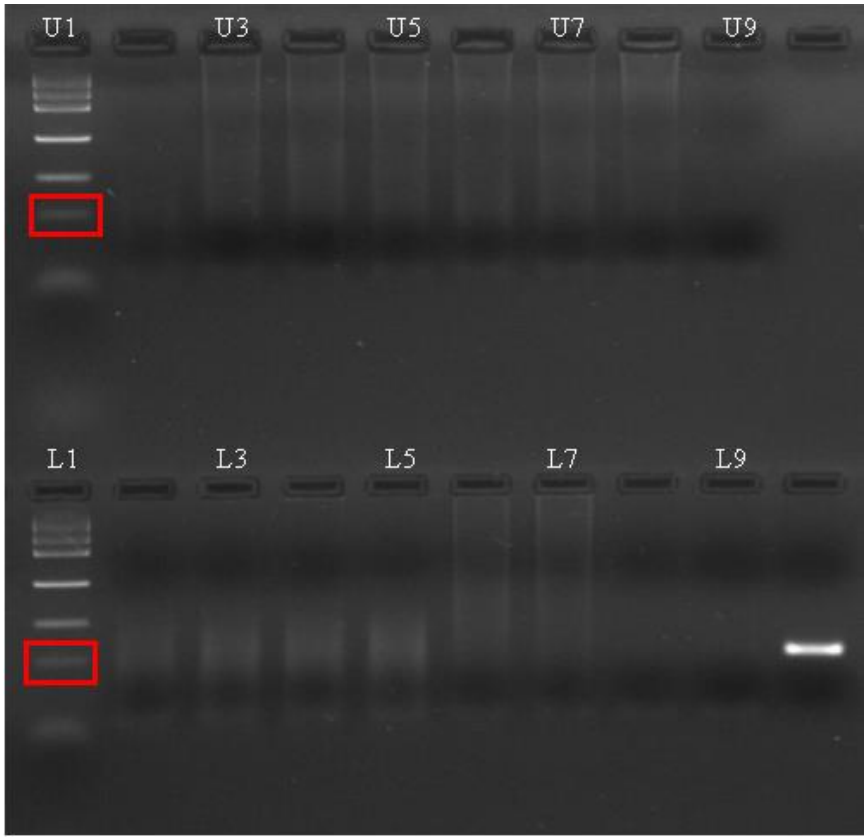

Figure 21: Agarose gel of (a portion of) January, 2014 field samples. 100 bp bands of ladders are outlined in red boxes (lanes U1 and L1). The target *L. salmonis* gene was 102 bp. Lane composition: L8 and L9 negative controls; L10 PCR positive; U10 blank; U2-U9 and L2-L7 miscellaneous field samples negative for the target gene.

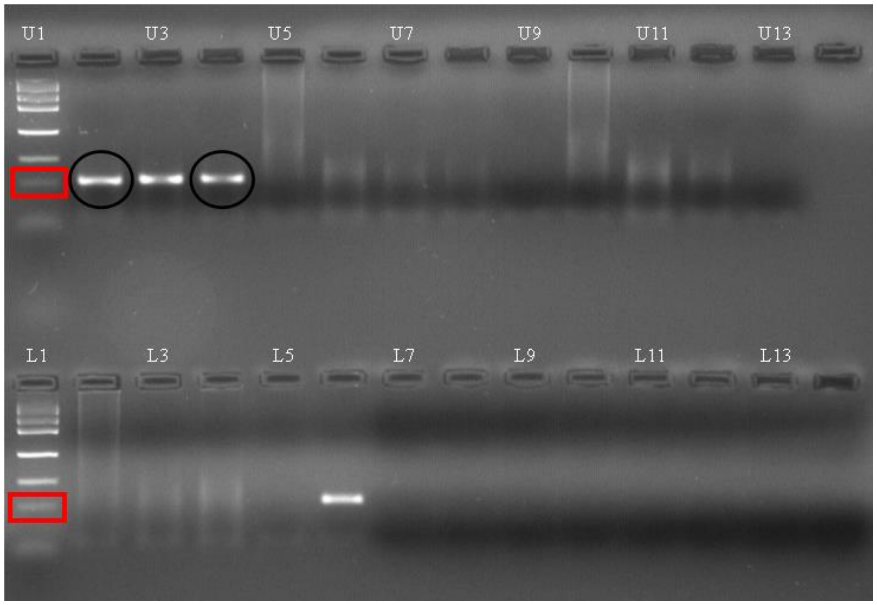

Figure 22: Agarose gel of (a portion of) January, 2014 field samples. 100 bp bands of ladders are outlined in red boxes (lanes U1 and L1). The target *L. salmonis* gene was 102 bp. Tissue positive (TP) and DNA positive samples are in U3 and L6, respectively. U9, U13, and L5 hold negative controls. U2 and U4 (circled) are field samples contaminated from the adjacent TP. Lanes L7-14 contain loading buffer only. Remaining lanes U5-U8, U10-U12, and L2-L4 hold field samples, negative for the target gene.

Appendix

Table 10: Raw data from lice counts, December 2012 through April 2014.

Year	Month	Pen	Fish #	Chalimus	*L. salmonis* pre-adult	*L. salmonis* male	*L. salmonis* female	*L. salmonis* gravid female	Motile *C. clemensi*
2012	December	4	1	0	0	1	1	1	1
2012	December	4	2	0	0	1	1	0	0
2012	December	4	3	0	1	0	0	0	1
2012	December	4	4	0	1	0	0	0	3
2012	December	4	5	0	0	0	0	0	1
2012	December	4	6	0	0	0	0	0	2
2012	December	4	7	0	0	0	0	0	4
2012	December	4	8	0	0	0	0	0	2
2012	December	4	9	0	0	0	0	0	0
2012	December	4	10	0	0	0	0	0	1
2012	December	4	11	0	0	0	0	0	1
2012	December	4	12	0	1	0	0	0	1
2012	December	4	13	0	0	1	2	0	0
2012	December	4	14	0	0	0	0	0	3
2012	December	4	15	0	1	1	0	1	1
2012	December	4	16	1	0	0	0	0	0
2012	December	4	17	0	0	0	0	0	3
2012	December	4	18	0	0	0	0	0	2
2012	December	4	19	0	0	0	0	0	2
2012	December	4	20	0	2	0	1	0	1
2012	December	12	1	0	0	0	0	0	0
2012	December	12	2	0	0	0	0	0	0
2012	December	12	3	0	0	0	0	0	0
2012	December	12	4	0	0	0	0	0	0
2012	December	12	5	0	0	0	0	0	1
2012	December	12	6	0	0	0	0	0	0
2012	December	12	7	0	1	0	0	0	2
2012	December	12	8	0	0	0	0	0	0
2012	December	12	9	0	0	1	0	0	1
2012	December	12	10	0	0	0	0	0	1
2012	December	12	11	0	0	0	0	0	0
2012	December	12	12	0	0	0	0	0	0
2012	December	12	13	0	0	0	0	0	1
2012	December	12	14	0	0	0	0	0	0
2012	December	12	15	0	1	0	0	0	0

2012	December	12	16	0	0	0	0	0	0
2012	December	12	17	0	0	0	0	0	0
2012	December	12	18	0	0	0	0	0	1
2012	December	12	19	0	0	0	0	0	0
2012	December	12	20	0	0	0	0	0	1
2012	December	1	1	0	0	0	1	0	1
2012	December	1	2	0	0	0	0	0	3
2012	December	1	3	0	0	0	0	0	1
2012	December	1	4	0	0	0	0	0	1
2012	December	1	5	0	1	0	0	0	1
2012	December	1	6	0	0	0	0	0	1
2012	December	1	7	0	0	0	0	0	1
2012	December	1	8	0	0	0	0	0	1
2012	December	1	9	0	1	0	0	0	3
2012	December	1	10	0	0	1	1	0	2
2012	December	1	11	0	0	0	1	0	2
2012	December	1	12	0	0	0	0	0	5
2012	December	1	13	0	0	0	0	0	1
2012	December	1	14	0	0	0	0	0	2
2012	December	1	15	0	0	0	0	0	4
2012	December	1	16	0	0	1	1	0	0
2012	December	1	17	0	0	1	1	0	2
2012	December	1	18	0	0	0	0	0	3
2012	December	1	19	0	0	0	1	0	1
2012	December	1	20	0	0	2	0	0	2
2012	December	2	1	0	0	0	1	1	0
2012	December	2	2	0	1	0	0	0	2
2012	December	2	3	0	1	1	0	0	1
2012	December	2	4	0	0	1	1	0	0
2012	December	2	5	0	0	0	0	0	1
2012	December	2	6	0	0	0	0	0	1
2012	December	2	7	0	0	0	0	0	2
2012	December	2	8	0	0	0	0	0	2
2012	December	2	9	0	0	0	1	0	0
2012	December	2	10	0	1	0	0	0	3
2012	December	2	11	0	0	0	0	0	3
2012	December	2	12	0	0	1	0	0	0
2012	December	2	13	0	1	0	0	0	1
2012	December	2	14	0	1	0	0	0	1
2012	December	2	15	0	0	0	0	1	1
2012	December	2	16	0	0	0	0	0	4

2012	December	2	17	0	0	0	0	0	2
2012	December	2	18	0	0	0	0	0	2
2012	December	2	19	0	0	0	0	0	3
2012	December	2	20	0	1	1	0	0	0
2012	December	3	1	0	0	0	2	1	1
2012	December	3	2	0	0	1	0	0	1
2012	December	3	3	0	0	0	0	0	3
2012	December	3	4	0	0	0	0	0	0
2012	December	3	5	0	0	0	0	0	2
2012	December	3	6	0	0	0	0	0	0
2012	December	3	7	0	0	1	0	0	2
2012	December	3	8	0	0	0	1	0	0
2012	December	3	9	0	1	0	0	0	1
2012	December	3	10	0	0	0	0	0	3
2012	December	3	11	0	1	0	0	0	0
2012	December	3	12	0	0	2	0	0	3
2012	December	3	13	1	0	0	0	0	1
2012	December	3	14	0	0	1	0	0	0
2012	December	3	15	1	1	0	0	0	0
2012	December	3	16	0	1	0	1	0	1
2012	December	3	17	0	0	1	0	0	0
2012	December	3	18	0	0	0	0	0	1
2012	December	3	19	0	0	1	0	0	1
2012	December	3	20	0	0	0	0	0	3
2012	December	10	1	0	0	0	0	0	0
2012	December	10	2	0	0	0	0	0	3
2012	December	10	3	0	1	0	0	0	1
2012	December	10	4	0	1	1	1	0	0
2012	December	10	5	0	0	0	0	0	0
2012	December	10	6	0	0	0	0	0	0
2012	December	10	7	0	0	0	0	0	1
2012	December	10	8	0	0	0	0	0	0
2012	December	10	9	0	0	0	0	0	0
2012	December	10	10	0	1	0	0	0	1
2012	December	10	11	0	0	0	0	0	0
2012	December	10	12	0	0	0	0	0	0
2012	December	10	13	0	1	0	0	0	3
2012	December	10	14	0	0	0	0	0	0
2012	December	10	15	0	0	0	0	0	0
2012	December	10	16	0	0	0	0	0	1
2012	December	10	17	0	0	0	0	0	0

2012	December	10	18	0	0	0	0	0	1
2012	December	10	19	0	0	0	0	0	0
2012	December	10	20	0	0	0	0	0	1
2013	January	6	1	0	0	0	0	0	2
2013	January	6	2	0	0	0	0	0	3
2013	January	6	3	0	0	0	0	0	8
2013	January	6	4	0	0	1	1	0	7
2013	January	6	5	0	0	0	0	0	2
2013	January	6	6	0	0	0	0	0	4
2013	January	6	7	0	0	0	0	0	1
2013	January	6	8	0	0	0	0	0	5
2013	January	6	9	0	0	0	1	0	2
2013	January	6	10	0	0	0	0	0	4
2013	January	6	11	0	0	1	0	0	18
2013	January	6	12	0	0	1	0	1	10
2013	January	6	13	0	0	1	0	1	6
2013	January	6	14	0	0	0	0	0	3
2013	January	6	15	0	0	1	0	0	5
2013	January	6	16	0	0	0	0	0	5
2013	January	6	17	0	0	1	0	0	6
2013	January	6	18	0	0	0	0	0	6
2013	January	6	19	0	0	0	1	3	8
2013	January	6	20	0	0	0	0	0	3
2013	January	7	1	0	0	0	0	0	3
2013	January	7	2	0	0	0	1	0	3
2013	January	7	3	0	0	0	1	0	4
2013	January	7	4	0	0	0	0	0	4
2013	January	7	5	0	0	0	0	0	5
2013	January	7	6	0	0	0	0	0	3
2013	January	7	7	0	0	0	0	1	2
2013	January	7	8	0	0	0	0	0	5
2013	January	7	9	0	0	0	0	0	2
2013	January	7	10	0	0	0	2	0	7
2013	January	7	11	0	0	0	0	0	2
2013	January	7	12	0	0	0	0	0	3
2013	January	7	13	0	0	1	0	0	3
2013	January	7	14	0	0	0	0	0	3
2013	January	7	15	0	0	0	0	0	1
2013	January	7	16	0	0	0	0	0	2
2013	January	7	17	0	0	0	0	0	2
2013	January	7	18	0	0	1	0	0	4

2013	January	7	19	0	0	0	0	0	5
2013	January	7	20	0	0	0	0	0	2
2013	January	3	1	0	0	0	0	0	7
2013	January	3	2	0	0	0	0	2	4
2013	January	3	3	0	0	1	0	0	1
2013	January	3	4	0	0	0	0	0	4
2013	January	3	5	0	0	0	0	0	3
2013	January	3	6	0	0	0	0	0	2
2013	January	3	7	0	0	0	1	1	3
2013	January	3	8	0	0	1	0	0	3
2013	January	3	9	0	0	0	0	0	1
2013	January	3	10	0	0	0	0	0	2
2013	January	3	11	0	0	1	1	2	5
2013	January	3	12	0	1	0	0	0	3
2013	January	3	13	0	0	0	0	0	4
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2013	January	3	15	0	0	0	0	0	2
2013	January	3	16	0	1	0	0	0	6
2013	January	3	17	0	1	0	0	0	4
2013	January	3	18	0	0	0	0	0	2
2013	January	3	19	0	0	0	0	0	4
2013	January	3	20	0	0	0	0	0	2
2013	January	1	1	0	0	1	1	0	8
2013	January	1	2	0	2	0	0	0	14
2013	January	1	3	0	1	0	0	0	4
2013	January	1	4	0	0	0	3	0	4
2013	January	1	5	0	0	0	0	0	13
2013	January	1	6	0	0	1	0	1	15
2013	January	1	7	0	0	0	0	0	5
2013	January	1	8	0	0	2	0	2	5
2013	January	1	9	0	0	1	0	0	3
2013	January	1	10	0	0	0	0	1	4
2013	January	1	11	0	0	0	0	0	12
2013	January	1	12	1	0	1	0	0	5
2013	January	1	13	0	0	1	1	0	1
2013	January	1	14	0	0	2	0	0	4
2013	January	1	15	0	1	0	0	0	2
2013	January	1	16	0	1	2	0	0	8
2013	January	1	17	0	0	1	0	0	1
2013	January	1	18	0	0	1	0	0	4
2013	January	1	19	1	1	2	0	0	8

2013	January	1	20	1	0	0	0	0	1
2013	January	2	1	1	0	0	0	0	1
2013	January	2	2	0	0	2	0	1	10
2013	January	2	3	0	0	1	0	0	5
2013	January	2	4	0	0	0	1	0	7
2013	January	2	5	0	0	0	0	0	2
2013	January	2	6	0	0	0	0	0	4
2013	January	2	7	0	0	0	0	0	0
2013	January	2	8	0	0	0	0	0	17
2013	January	2	9	0	0	1	1	0	4
2013	January	2	10	1	0	0	0	2	9
2013	January	2	11	0	0	0	0	0	7
2013	January	2	12	0	0	0	0	0	11
2013	January	2	13	0	0	0	0	0	9
2013	January	2	14	0	0	1	1	0	4
2013	January	2	15	0	1	0	1	0	9
2013	January	2	16	0	0	0	0	0	10
2013	January	2	17	0	1	2	0	0	3
2013	January	2	18	0	0	2	2	0	18
2013	January	2	19	0	0	2	0	0	18
2013	January	2	20	0	0	1	1	0	11
2013	January	8	1	0	0	0	0	0	5
2013	January	8	2	0	0	0	0	0	3
2013	January	8	3	0	0	0	0	0	1
2013	January	8	4	0	0	4	0	1	12
2013	January	8	5	0	0	0	0	0	3
2013	January	8	6	0	0	0	0	0	1
2013	January	8	7	0	0	1	0	0	0
2013	January	8	8	0	0	1	0	1	23
2013	January	8	9	0	0	0	1	0	3
2013	January	8	10	2	0	2	0	0	7
2013	January	8	11	3	0	1	0	0	6
2013	January	8	12	0	0	0	0	0	2
2013	January	8	13	0	0	0	0	0	5
2013	January	8	14	1	0	0	1	0	3
2013	January	8	15	1	0	0	0	0	9
2013	January	8	16	0	2	1	1	0	23
2013	January	8	17	0	0	0	0	0	6
2013	January	8	18	1	1	0	0	1	10
2013	January	8	19	0	0	2	0	0	10
2013	January	8	20	0	0	0	0	0	4

2013	February	9	1	0	2	0	0	0	3
2013	February	9	2	0	0	0	0	0	0
2013	February	9	3	0	0	0	0	0	0
2013	February	9	4	0	0	0	0	0	0
2013	February	9	5	0	0	0	0	0	4
2013	February	9	6	0	0	0	0	0	1
2013	February	9	7	0	0	0	0	0	2
2013	February	9	8	0	0	0	0	0	2
2013	February	9	9	0	0	0	0	0	1
2013	February	9	10	0	0	0	0	0	2
2013	February	9	11	0	0	0	0	0	0
2013	February	9	12	0	1	0	0	0	1
2013	February	9	13	0	0	0	0	0	1
2013	February	9	14	0	0	0	0	0	1
2013	February	9	15	0	0	0	0	0	0
2013	February	9	16	0	0	0	0	0	0
2013	February	9	17	0	0	0	0	0	0
2013	February	9	18	0	1	0	0	0	3
2013	February	9	19	0	0	0	0	0	0
2013	February	9	20	0	0	0	0	0	2
2013	February	3	1	0	0	0	0	0	5
2013	February	3	2	0	1	0	0	1	3
2013	February	3	3	0	0	0	0	0	2
2013	February	3	4	0	2	0	0	1	2
2013	February	3	5	0	0	0	0	0	2
2013	February	3	6	0	0	0	0	0	1
2013	February	3	7	0	2	0	0	0	3
2013	February	3	8	0	2	0	0	0	2
2013	February	3	9	0	0	1	0	1	5
2013	February	3	10	0	0	1	0	0	5
2013	February	3	11	0	0	0	0	1	0
2013	February	3	12	0	1	0	0	0	1
2013	February	3	13	0	0	0	0	0	1
2013	February	3	14	0	1	0	0	2	1
2013	February	3	15	0	2	0	0	0	1
2013	February	3	16	0	1	0	0	0	2
2013	February	3	17	0	0	0	1	0	3
2013	February	3	18	0	0	0	0	0	0
2013	February	3	19	0	0	0	0	1	0
2013	February	3	20	0	1	0	0	0	1
2013	February	5	1	0	0	0	0	0	2

2013	February	5	2	0	0	0	0	0	1
2013	February	5	3	0	0	0	0	0	1
2013	February	5	4	0	1	0	0	0	0
2013	February	5	5	0	1	0	0	0	1
2013	February	5	6	0	0	0	0	0	0
2013	February	5	7	0	3	0	0	0	3
2013	February	5	8	0	0	0	0	0	1
2013	February	5	9	0	1	0	0	0	0
2013	February	5	10	0	0	0	0	0	2
2013	February	5	11	0	0	0	0	0	2
2013	February	5	12	0	0	0	0	0	1
2013	February	5	13	0	0	0	0	1	5
2013	February	5	14	0	0	0	0	0	0
2013	February	5	15	0	0	0	0	0	2
2013	February	5	16	0	0	0	0	0	1
2013	February	5	17	0	0	0	0	0	1
2013	February	5	18	0	2	0	0	0	3
2013	February	5	19	0	0	0	0	0	1
2013	February	5	20	0	1	0	0	0	1
2013	March	3	1	0	0	0	0	0	0
2013	March	3	2	0	0	0	0	0	0
2013	March	3	3	0	0	0	0	0	0
2013	March	3	4	0	0	0	0	0	0
2013	March	3	5	0	0	0	0	0	0
2013	March	3	6	0	0	0	0	0	0
2013	March	3	7	0	1	0	0	0	0
2013	March	3	8	0	0	0	0	0	1
2013	March	3	9	0	0	0	0	0	0
2013	March	3	10	0	0	0	0	0	0
2013	March	3	11	0	0	0	0	0	0
2013	March	3	12	0	0	0	0	0	0
2013	March	3	13	0	0	0	0	0	0
2013	March	3	14	0	0	0	0	0	0
2013	March	3	15	0	0	0	0	0	0
2013	March	3	16	0	0	0	0	0	0
2013	March	3	17	0	0	0	0	0	0
2013	March	3	18	0	0	0	0	0	0
2013	March	3	19	0	0	0	0	0	0
2013	March	3	20	0	0	0	0	0	0
2013	March	11	1	0	0	0	0	0	0
2013	March	11	2	0	0	0	0	0	0

2013	March	11	3	0	0	0	0	0	0
2013	March	11	4	0	0	0	0	0	0
2013	March	11	5	0	0	0	0	0	0
2013	March	11	6	0	0	0	0	0	0
2013	March	11	7	0	0	0	0	0	0
2013	March	11	8	0	0	0	1	0	0
2013	March	11	9	0	0	0	1	0	0
2013	March	11	10	0	0	0	0	0	0
2013	March	11	11	0	0	0	0	0	0
2013	March	11	12	0	0	0	0	0	0
2013	March	11	13	0	0	0	0	0	0
2013	March	11	14	0	0	0	0	0	0
2013	March	11	15	0	0	0	1	0	0
2013	March	11	16	0	0	0	0	0	0
2013	March	11	17	0	0	0	0	0	0
2013	March	11	18	0	0	0	0	0	0
2013	March	11	19	0	0	0	0	0	0
2013	March	11	20	0	0	0	0	0	0
2013	March	13	1	0	0	0	0	0	0
2013	March	13	2	0	0	0	0	0	0
2013	March	13	3	0	0	0	0	0	0
2013	March	13	4	0	0	0	0	0	1
2013	March	13	5	0	0	0	0	0	0
2013	March	13	6	0	0	0	0	0	0
2013	March	13	7	0	0	0	0	0	0
2013	March	13	8	0	0	0	0	0	0
2013	March	13	9	0	0	0	1	0	0
2013	March	13	10	0	0	0	0	0	0
2013	March	13	11	0	0	0	0	0	0
2013	March	13	12	0	0	0	0	0	0
2013	March	13	13	0	0	0	0	0	0
2013	March	13	14	0	0	0	0	0	0
2013	March	13	15	0	0	0	0	0	0
2013	March	13	16	0	0	0	0	0	0
2013	March	13	17	0	0	0	0	0	0
2013	March	13	18	0	0	0	0	0	0
2013	March	13	19	0	0	0	0	0	0
2013	March	13	20	0	0	0	0	0	0
2013	March	14	1	0	0	0	0	0	0
2013	March	14	2	0	0	0	0	0	0
2013	March	14	3	0	0	0	0	0	0

2013	March	14	4	0	0	0	0	0	0
2013	March	14	5	0	0	0	0	0	0
2013	March	14	6	0	0	0	0	0	0
2013	March	14	7	0	0	0	0	0	0
2013	March	14	8	0	0	0	0	0	0
2013	March	14	9	0	0	0	0	0	0
2013	March	14	10	0	0	0	0	0	0
2013	March	14	11	0	0	0	0	0	0
2013	March	14	12	0	0	0	0	0	0
2013	March	14	13	0	0	0	0	0	0
2013	March	14	14	0	0	0	0	0	0
2013	March	14	15	0	0	0	0	0	0
2013	March	14	16	0	0	0	0	0	0
2013	March	14	17	0	0	0	0	0	0
2013	March	14	18	0	0	0	0	0	0
2013	March	14	19	0	0	0	0	0	0
2013	March	14	20	0	0	0	0	0	0
2013	March	12	1	0	0	0	0	0	0
2013	March	12	2	0	0	0	0	0	0
2013	March	12	3	0	0	0	0	0	0
2013	March	12	4	0	0	0	0	0	0
2013	March	12	5	0	0	0	0	0	0
2013	March	12	6	0	0	0	0	0	0
2013	March	12	7	0	0	0	0	0	4
2013	March	12	8	0	0	0	0	0	0
2013	March	12	9	0	0	0	0	0	0
2013	March	12	10	0	0	0	0	0	0
2013	March	12	11	0	0	0	0	0	0
2013	March	12	12	0	0	0	0	0	0
2013	March	12	13	0	0	0	0	0	0
2013	March	12	14	0	0	0	0	0	2
2013	March	12	15	0	0	0	0	0	0
2013	March	12	16	0	0	0	0	0	0
2013	March	12	17	0	0	0	0	0	0
2013	March	12	18	0	0	0	0	0	0
2013	March	12	19	0	0	0	0	0	0
2013	March	12	20	0	0	0	0	0	0
2013	March	3 (again)	1	0	0	0	0	0	0
2013	March	3	2	0	0	0	0	0	0
2013	March	3	3	0	0	0	0	0	0
2013	March	3	4	0	0	0	0	0	1

2013	March	3	5	0	0	0	0	0	0
2013	March	3	6	0	0	0	0	0	0
2013	March	3	7	0	0	0	0	0	0
2013	March	3	8	0	0	0	0	0	0
2013	March	3	9	0	0	0	0	0	0
2013	March	3	10	0	0	0	0	0	0
2013	March	3	11	0	0	0	0	0	0
2013	March	3	12	0	0	0	0	0	0
2013	March	3	13	0	0	0	0	0	0
2013	March	3	14	0	0	0	0	0	0
2013	March	3	15	0	0	0	0	0	0
2013	March	3	16	0	0	0	0	0	0
2013	March	3	17	0	0	0	0	0	0
2013	March	3	18	0	0	0	0	0	0
2013	March	3	19	0	0	0	0	0	0
2013	March	3	20	0	0	0	0	0	0
2013	April	3	1	0	0	0	0	0	0
2013	April	3	2	0	0	0	0	0	0
2013	April	3	3	0	0	0	0	0	0
2013	April	3	4	0	0	0	0	0	0
2013	April	3	5	0	0	0	0	0	0
2013	April	3	6	0	0	0	0	0	0
2013	April	3	7	0	0	1	0	0	0
2013	April	3	8	0	0	0	0	0	0
2013	April	3	9	0	0	0	0	0	0
2013	April	3	10	0	0	0	0	0	0
2013	April	3	11	0	0	0	0	0	0
2013	April	3	12	0	0	0	0	0	0
2013	April	3	13	0	0	0	0	0	0
2013	April	3	14	0	0	0	0	0	0
2013	April	3	15	0	0	0	0	0	0
2013	April	3	16	0	0	0	0	0	0
2013	April	3	17	0	0	0	0	0	1
2013	April	3	18	0	0	0	0	0	0
2013	April	3	19	0	0	0	0	0	1
2013	April	3	20	0	0	0	0	0	0
2013	April	4	1	0	2	3	1	3	3
2013	April	4	2	0	0	0	0	0	0
2013	April	4	3	0	0	0	0	0	2
2013	April	4	4	0	0	0	0	0	0
2013	April	4	5	0	0	1	0	2	0

2013	April	4	6	0	0	1	0	0	0
2013	April	4	7	0	0	0	0	0	4
2013	April	4	8	0	0	0	0	0	1
2013	April	4	9	0	0	0	0	0	0
2013	April	4	10	0	0	0	0	0	0
2013	April	4	11	0	0	0	0	0	0
2013	April	4	12	0	0	0	0	0	0
2013	April	4	13	0	0	0	0	0	0
2013	April	4	14	0	0	1	0	0	0
2013	April	4	15	0	0	0	0	0	0
2013	April	4	16	0	0	0	0	0	0
2013	April	4	17	0	0	0	0	0	0
2013	April	4	18	0	0	0	0	0	0
2013	April	4	19	0	0	1	0	0	0
2013	April	4	20	0	0	0	0	0	0
2013	April	6	1	0	0	0	0	0	0
2013	April	6	2	0	0	0	0	1	0
2013	April	6	3	0	0	0	0	0	0
2013	April	6	4	0	0	0	0	0	4
2013	April	6	5	0	0	0	0	0	0
2013	April	6	6	0	0	0	0	0	0
2013	April	6	7	0	0	0	0	0	0
2013	April	6	8	0	0	0	0	0	3
2013	April	6	9	0	0	0	0	1	1
2013	April	6	10	0	0	0	0	0	0
2013	April	6	11	0	1	0	0	0	2
2013	April	6	12	0	0	0	0	0	0
2013	April	6	13	0	0	0	0	0	0
2013	April	6	14	0	0	0	0	0	0
2013	April	6	15	0	0	0	0	0	0
2013	April	6	16	0	1	0	0	0	2
2013	April	6	17	0	0	0	1	0	0
2013	April	6	18	0	0	0	0	0	0
2013	April	6	19	0	0	0	0	0	1
2013	April	6	20	0	0	0	0	0	0
2013	April	3	1	0	0	0	0	0	0
2013	April	3	2	0	0	0	0	0	0
2013	April	3	3	0	0	0	0	0	0
2013	April	3	4	0	0	0	0	0	0
2013	April	3	5	0	0	0	0	0	0
2013	April	3	6	0	0	0	0	0	0

2013	April	3	7	0	0	0	0	0	0
2013	April	3	8	0	0	0	0	0	0
2013	April	3	9	0	0	0	0	0	0
2013	April	3	10	0	0	0	0	0	0
2013	April	3	11	0	0	0	0	0	0
2013	April	3	12	0	0	0	0	0	1
2013	April	3	13	0	0	0	0	0	0
2013	April	3	14	0	0	0	0	0	0
2013	April	3	15	0	0	0	0	0	0
2013	April	3	16	0	0	0	0	0	0
2013	April	3	17	0	0	0	0	0	0
2013	April	3	18	0	0	0	0	0	0
2013	April	3	19	0	0	0	0	0	0
2013	April	3	20	0	0	0	0	0	0
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2013	April	1	4	0	0	0	0	0	0
2013	April	1	5	0	0	0	0	0	0
2013	April	1	6	0	0	0	0	0	0
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2013	April	1	8	0	0	0	0	0	0
2013	April	1	9	0	0	0	0	0	0
2013	April	1	10	0	0	0	0	0	0
2013	April	1	11	0	0	0	0	0	0
2013	April	1	12	0	0	0	0	0	1
2013	April	1	13	0	0	0	0	0	0
2013	April	1	14	0	0	0	0	0	0
2013	April	1	15	0	0	0	0	0	0
2013	April	1	16	0	0	0	0	0	0
2013	April	1	17	0	0	0	0	0	0
2013	April	1	18	0	0	0	0	0	0
2013	April	1	19	0	0	0	0	0	0
2013	April	1	20	0	0	0	1	0	0
2013	April	2	1	0	0	0	0	0	0
2013	April	2	2	0	0	0	0	1	0
2013	April	2	3	0	0	0	0	0	0
2013	April	2	4	0	0	0	0	0	0
2013	April	2	5	0	0	0	0	0	0
2013	April	2	6	0	0	0	0	0	0
2013	April	2	7	0	0	0	0	0	0

2013	April	2	8	0	0	0	0	0	0
2013	April	2	9	0	0	0	0	0	0
2013	April	2	10	0	0	0	0	0	0
2013	April	2	11	0	0	0	0	0	0
2013	April	2	12	0	0	0	0	0	0
2013	April	2	13	0	0	0	0	0	0
2013	April	2	14	0	0	0	0	0	0
2013	April	2	15	0	0	0	0	0	0
2013	April	2	16	0	0	0	0	0	0
2013	April	2	17	0	0	0	0	0	0
2013	April	2	18	0	0	0	0	0	0
2013	April	2	19	0	0	0	0	0	1
2013	April	2	20	0	0	0	0	0	1
2013	May	9	1	0	0	0	0	0	0
2013	May	9	2	0	0	0	0	0	0
2013	May	9	3	0	0	0	0	0	0
2013	May	9	4	0	0	0	0	0	0
2013	May	9	5	0	0	0	0	0	0
2013	May	9	6	0	0	0	0	0	0
2013	May	9	7	0	0	0	0	0	0
2013	May	9	8	0	0	0	0	0	0
2013	May	9	9	0	0	0	0	0	0
2013	May	9	10	0	0	0	0	0	0
2013	May	9	11	0	0	0	0	0	0
2013	May	9	12	0	0	0	0	0	0
2013	May	9	13	0	0	0	0	0	0
2013	May	9	14	0	0	0	0	0	0
2013	May	9	15	0	0	0	0	0	0
2013	May	9	16	0	0	0	0	0	0
2013	May	9	17	0	0	0	0	0	0
2013	May	9	18	0	0	0	0	0	0
2013	May	9	19	0	0	0	0	0	0
2013	May	9	20	0	0	0	0	0	0
2013	May	3	1	0	0	0	0	0	0
2013	May	3	2	0	0	0	0	0	0
2013	May	3	3	0	0	0	0	0	0
2013	May	3	4	0	0	0	0	0	0
2013	May	3	5	0	0	0	0	0	0
2013	May	3	6	0	0	0	0	0	0
2013	May	3	7	0	0	0	0	0	0
2013	May	3	8	0	0	0	0	0	0

2013	May	3	9	0	0	0	0	0	0
2013	May	3	10	0	0	0	0	0	0
2013	May	3	11	0	0	0	0	0	0
2013	May	3	12	0	0	0	0	0	0
2013	May	3	13	0	0	0	0	0	0
2013	May	3	14	0	0	0	0	0	0
2013	May	3	15	0	0	1	0	0	0
2013	May	3	16	0	0	0	0	0	0
2013	May	3	17	0	0	0	0	0	0
2013	May	3	18	0	0	0	0	0	0
2013	May	3	19	0	0	0	0	0	0
2013	May	3	20	0	0	0	0	0	0
2013	May	5	1	0	0	0	0	0	0
2013	May	5	2	0	0	0	0	0	0
2013	May	5	3	0	0	0	0	0	0
2013	May	5	4	0	1	0	0	0	0
2013	May	5	5	0	0	0	0	0	0
2013	May	5	6	0	0	0	0	0	0
2013	May	5	7	0	0	0	0	0	0
2013	May	5	8	0	0	0	0	0	0
2013	May	5	9	0	0	0	0	0	0
2013	May	5	10	0	0	0	0	0	0
2013	May	5	11	0	0	0	0	0	0
2013	May	5	12	0	0	0	0	0	0
2013	May	5	13	0	0	0	0	0	0
2013	May	5	14	0	0	0	0	0	0
2013	May	5	15	0	0	0	1	0	0
2013	May	5	16	0	0	0	0	0	0
2013	May	5	17	0	0	0	0	0	0
2013	May	5	18	0	0	0	0	0	0
2013	May	5	19	0	0	0	0	0	0
2013	May	5	20	0	0	0	0	0	0
2013	May	3	1	0	0	0	0	0	0
2013	May	3	2	0	0	0	0	0	0
2013	May	3	3	0	0	0	0	0	0
2013	May	3	4	0	0	0	0	0	0
2013	May	3	5	0	0	0	0	0	0
2013	May	3	6	0	0	0	0	0	0
2013	May	3	7	0	1	0	0	0	0
2013	May	3	8	0	0	0	0	0	0
2013	May	3	9	0	0	0	0	0	0

2013	May	3	10	0	0	0	0	0	0
2013	May	3	11	0	0	0	0	0	0
2013	May	3	12	0	0	0	0	0	0
2013	May	3	13	0	0	0	0	0	0
2013	May	3	14	0	0	0	0	0	0
2013	May	3	15	0	0	0	0	0	0
2013	May	3	16	0	0	0	0	0	0
2013	May	3	17	0	0	0	0	0	0
2013	May	3	18	0	0	0	0	0	0
2013	May	3	19	0	0	0	0	0	0
2013	May	3	20	0	0	0	0	0	0
2013	May	11	1	0	0	0	0	0	0
2013	May	11	2	0	0	0	0	0	0
2013	May	11	3	0	0	0	0	0	0
2013	May	11	4	0	0	0	0	0	0
2013	May	11	5	0	0	0	0	0	0
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2013	May	11	9	0	0	0	0	0	0
2013	May	11	10	0	0	0	0	0	0
2013	May	11	11	0	0	0	0	0	0
2013	May	11	12	0	0	0	0	0	0
2013	May	11	13	0	0	0	0	0	1
2013	May	11	14	0	0	0	0	0	0
2013	May	11	15	0	0	0	0	0	0
2013	May	11	16	0	0	0	0	0	0
2013	May	11	17	0	0	0	0	0	0
2013	May	11	18	0	0	0	0	0	0
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2013	May	11	20	1	0	0	0	0	0
2013	May	13	1	0	0	0	0	0	0
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2013	May	13	5	0	0	0	0	0	0
2013	May	13	6	0	0	0	0	0	0
2013	May	13	7	0	0	0	0	0	0
2013	May	13	8	0	0	0	0	0	1
2013	May	13	9	0	0	0	0	0	0
2013	May	13	10	0	0	0	0	0	0

2013	May	13	11	0	0	0	0	0	0
2013	May	13	12	0	0	0	0	0	0
2013	May	13	13	0	0	0	0	0	0
2013	May	13	14	0	0	0	0	0	2
2013	May	13	15	0	0	0	0	0	0
2013	May	13	16	0	0	0	0	0	0
2013	May	13	17	0	0	0	0	0	1
2013	May	13	18	0	0	0	0	0	0
2013	May	13	19	0	0	0	0	0	0
2013	May	13	20	0	0	0	0	0	0
2013	June	3	1	0	0	0	0	0	0
2013	June	3	2	0	0	0	0	0	0
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2013	June	3	6	0	0	0	0	0	0
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2013	June	3	8	0	0	0	0	0	0
2013	June	3	9	0	0	0	0	0	0
2013	June	3	10	0	0	0	0	0	0
2013	June	3	11	0	0	0	0	0	0
2013	June	3	12	0	0	0	0	0	0
2013	June	3	13	0	0	0	0	0	0
2013	June	3	14	0	0	0	0	0	0
2013	June	3	15	0	0	0	0	0	0
2013	June	3	16	0	0	0	0	0	0
2013	June	3	17	0	0	0	0	0	1
2013	June	3	18	0	0	0	0	0	0
2013	June	3	19	0	0	0	0	0	0
2013	June	3	20	0	0	0	0	0	0
2013	June	7	1	0	0	0	0	0	0
2013	June	7	2	0	0	0	0	0	0
2013	June	7	3	0	0	0	0	0	0
2013	June	7	4	0	0	0	0	0	0
2013	June	7	5	0	0	0	0	0	0
2013	June	7	6	0	0	0	0	0	0
2013	June	7	7	0	0	0	0	0	0
2013	June	7	8	0	0	0	0	0	0
2013	June	7	9	0	0	0	0	0	0
2013	June	7	10	0	0	0	0	0	0
2013	June	7	11	0	0	0	0	0	0

2013	June	7	12	0	0	0	0	0	0
2013	June	7	13	0	0	0	0	0	0
2013	June	7	14	0	0	0	0	0	0
2013	June	7	15	0	0	0	0	0	0
2013	June	7	16	0	0	0	0	0	0
2013	June	7	17	0	0	0	0	0	0
2013	June	7	18	0	0	0	0	0	0
2013	June	7	19	0	0	0	0	0	1
2013	June	7	20	0	0	0	0	0	0
2013	June	8	1	0	0	0	0	0	0
2013	June	8	2	0	0	0	0	0	0
2013	June	8	3	0	0	0	0	0	0
2013	June	8	4	0	0	0	0	0	0
2013	June	8	5	0	0	0	0	0	2
2013	June	8	6	0	0	0	0	0	0
2013	June	8	7	0	0	0	0	0	0
2013	June	8	8	0	0	0	0	0	0
2013	June	8	9	0	0	0	0	0	0
2013	June	8	10	0	0	0	0	0	0
2013	June	8	11	0	0	0	0	0	0
2013	June	8	12	0	0	0	0	0	0
2013	June	8	13	0	0	0	0	0	0
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2013	June	8	15	0	0	0	0	0	0
2013	June	8	16	0	0	0	0	0	0
2013	June	8	17	0	0	0	0	0	0
2013	June	8	18	0	0	0	0	0	0
2013	June	8	19	0	0	0	0	0	0
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2013	June	14	5	0	0	0	0	0	0
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2013	June	14	7	0	0	0	0	0	0
2013	June	14	8	0	0	0	0	0	0
2013	June	14	9	0	0	0	0	0	0
2013	June	14	10	0	0	0	0	0	0
2013	June	14	11	0	0	0	0	0	0
2013	June	14	12	0	0	0	0	0	0

2013	June	14	13	0	0	0	0	0	0
2013	June	14	14	0	0	0	0	0	0
2013	June	14	15	0	0	0	0	0	0
2013	June	14	16	0	0	0	0	0	0
2013	June	14	17	0	0	0	0	0	0
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2013	June	12	10	0	0	0	0	0	0
2013	June	12	11	0	0	0	0	0	0
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2013	June	3	1	0	0	0	0	0	0
2013	June	3	2	0	0	0	0	0	1
2013	June	3	3	0	0	0	0	0	0
2013	June	3	4	0	0	0	0	0	0
2013	June	3	5	0	0	0	0	0	0
2013	June	3	6	0	0	0	0	0	0
2013	June	3	7	0	0	0	0	0	0
2013	June	3	8	0	0	0	0	0	1
2013	June	3	9	0	0	0	0	0	0
2013	June	3	10	0	0	0	0	0	0
2013	June	3	11	0	0	0	0	0	0
2013	June	3	12	0	0	0	0	0	0
2013	June	3	13	0	0	0	0	0	0

2013	June	3	14	0	0	0	0	0	0
2013	June	3	15	0	0	0	0	0	0
2013	June	3	16	0	0	0	0	0	0
2013	June	3	17	0	0	0	0	0	0
2013	June	3	18	0	0	0	0	0	0
2013	June	3	19	0	0	0	0	0	0
2013	June	3	20	0	0	0	0	0	0
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2013	July	4	2	0	0	0	0	0	0
2013	July	4	3	0	0	0	0	0	1
2013	July	4	4	0	0	0	0	0	0
2013	July	4	5	0	0	0	0	0	0
2013	July	4	6	0	0	0	0	0	0
2013	July	4	7	0	0	0	0	0	0
2013	July	4	8	0	0	1	0	0	0
2013	July	4	9	0	0	0	0	0	0
2013	July	4	10	0	0	0	0	0	0
2013	July	4	11	0	0	0	0	0	0
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2013	July	4	15	0	0	0	0	0	0
2013	July	4	16	0	0	0	0	0	0
2013	July	4	17	0	0	0	0	0	0
2013	July	4	18	0	0	0	0	0	0
2013	July	4	19	0	0	0	0	0	0
2013	July	4	20	0	0	1	0	0	0
2013	July	6	1	0	0	0	0	0	0
2013	July	6	2	0	0	0	0	0	0
2013	July	6	3	0	0	0	0	0	0
2013	July	6	4	0	0	0	0	0	0
2013	July	6	5	0	0	0	0	0	0
2013	July	6	6	0	0	0	0	0	0
2013	July	6	7	0	1	0	0	0	0
2013	July	6	8	0	0	0	0	0	0
2013	July	6	9	0	0	0	0	0	0
2013	July	6	10	0	0	0	0	0	0
2013	July	6	11	0	0	0	0	0	0
2013	July	6	12	0	0	0	0	0	0
2013	July	6	13	0	0	0	0	0	0
2013	July	6	14	0	0	0	0	0	0

2013	July	6	15	0	0	0	0	0	0
2013	July	6	16	0	0	0	0	0	0
2013	July	6	17	0	0	0	0	0	0
2013	July	6	18	0	0	0	0	0	0
2013	July	6	19	0	0	0	0	1	0
2013	July	6	20	0	0	0	0	0	0
2013	August	1	1	0	0	0	0	0	0
2013	August	1	2	0	0	0	0	0	0
2013	August	1	3	0	0	0	0	0	0
2013	August	1	4	0	0	0	0	0	0
2013	August	1	5	0	0	0	0	0	0
2013	August	1	6	0	0	0	0	0	0
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2013	August	1	9	0	0	0	0	0	0
2013	August	1	10	0	0	0	0	0	0
2013	August	1	11	0	0	0	0	0	0
2013	August	1	12	0	0	0	0	0	0
2013	August	1	13	0	0	0	0	0	0
2013	August	1	14	0	0	0	0	0	0
2013	August	1	15	0	0	0	0	0	0
2013	August	1	16	0	0	0	0	0	0
2013	August	1	17	0	0	0	0	0	0
2013	August	1	18	0	0	0	0	0	0
2013	August	1	19	0	0	0	0	1	0
2013	August	1	20	0	0	0	0	0	0
2013	August	3	1	0	0	0	0	0	0
2013	August	3	2	0	0	0	0	0	0
2013	August	3	3	0	0	0	0	0	0
2013	August	3	4	0	0	0	0	0	0
2013	August	3	5	0	0	0	0	0	0
2013	August	3	6	0	0	0	0	0	0
2013	August	3	7	0	0	0	0	0	0
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2013	August	3	9	0	0	0	0	0	0
2013	August	3	10	0	0	0	0	0	1
2013	August	3	11	0	0	0	0	0	0
2013	August	3	12	0	0	0	0	0	0
2013	August	3	13	0	0	0	0	0	0
2013	August	3	14	0	0	0	0	0	0
2013	August	3	15	0	0	0	0	0	0

2013	August	3	16	0	1	0	0	0	0
2013	August	3	17	0	0	0	0	0	0
2013	August	3	18	0	0	0	0	0	0
2013	August	3	19	0	0	0	0	0	0
2013	August	3	20	0	0	0	0	0	0
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2013	September	3	8	0	0	0	0	0	0
2013	September	3	9	0	0	0	0	0	0
2013	September	3	10	0	0	0	0	0	0
2013	September	3	11	0	0	1	1	0	0
2013	September	3	12	0	0	0	0	0	0
2013	September	3	13	0	0	0	0	0	0
2013	September	3	14	0	0	0	1	0	0
2013	September	3	15	0	0	0	0	0	0
2013	September	3	16	0	1	0	0	0	0

2013	September	3	17	0	0	0	1	0	0
2013	September	3	18	0	0	0	0	0	0
2013	September	3	19	0	0	0	0	0	0
2013	September	3	20	0	0	0	0	0	0
2013	September	1	1	0	0	0	0	0	0
2013	September	1	2	2	0	0	0	0	0
2013	September	1	3	1	0	0	0	0	0
2013	September	1	4	0	0	0	0	0	0
2013	September	1	5	0	0	1	0	0	0
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2013	September	1	7	0	0	0	0	0	0
2013	September	1	8	0	0	0	0	0	0
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2013	September	1	10	0	0	0	0	0	0
2013	September	1	11	0	0	0	0	0	0
2013	September	1	12	0	0	1	0	0	0
2013	September	1	13	0	0	0	0	1	0
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2013	September	1	15	0	0	1	0	1	0
2013	September	1	16	0	0	0	1	0	0
2013	September	1	17	0	0	1	1	1	0
2013	September	1	18	0	1	0	0	0	0
2013	September	1	19	1	0	0	0	0	0
2013	September	1	20	0	0	0	0	1	1
2013	November	1	1	0	0	0	0	0	0
2013	November	1	2	2	0	0	0	0	0
2013	November	1	3	1	0	0	0	0	0
2013	November	1	4	0	0	0	0	0	0
2013	November	1	5	0	0	1	0	0	0
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2013	November	1	7	0	0	0	0	0	0
2013	November	1	8	0	0	0	0	0	0
2013	November	1	9	0	1	0	0	0	0
2013	November	1	10	0	0	0	0	0	0
2013	November	1	11	0	0	0	0	0	0
2013	November	1	12	0	0	1	0	0	0
2013	November	1	13	0	0	0	0	1	0
2013	November	1	14	0	1	0	0	0	0
2013	November	1	15	0	0	1	0	1	0
2013	November	1	16	0	0	0	1	0	0
2013	November	1	17	0	0	1	1	1	0

2013	November	1	18	0	1	0	0	0	0
2013	November	1	19	1	0	0	0	0	0
2013	November	1	20	0	0	0	0	1	1
2013	November	3	1	0	0	0	0	0	0
2013	November	3	2	0	0	0	0	1	0
2013	November	3	3	0	0	1	0	0	0
2013	November	3	4	0	0	0	0	0	0
2013	November	3	5	0	0	0	0	0	0
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2013	November	3	11	0	0	0	0	0	0
2013	November	3	12	0	0	0	0	0	0
2013	November	3	13	0	0	0	1	0	0
2013	November	3	14	0	0	0	0	0	0
2013	November	3	15	0	0	0	0	0	0
2013	November	3	16	1	0	0	0	0	0
2013	November	3	17	0	0	0	0	0	0
2013	November	3	18	0	0	0	0	0	0
2013	November	3	19	0	0	0	0	0	0
2013	November	3	20	0	0	0	0	1	0
2013	November	14	1	0	0	1	0	1	1
2013	November	14	2	0	0	1	1	0	0
2013	November	14	3	0	0	0	0	3	2
2013	November	14	4	0	0	1	0	1	1
2013	November	14	5	0	0	0	1	2	0
2013	November	14	6	0	0	0	0	0	1
2013	November	14	7	0	0	0	0	1	0
2013	November	14	8	0	0	1	0	1	2
2013	November	14	9	0	0	0	0	0	1
2013	November	14	10	0	0	0	0	0	0
2013	November	14	11	2	1	1	0	0	1
2013	November	14	12	0	1	0	1	1	0
2013	November	14	13	0	0	0	0	0	0
2013	November	14	14	0	0	2	1	0	0
2013	November	14	15	0	1	0	0	2	0
2013	November	14	16	0	0	0	1	1	2
2013	November	14	17	0	1	0	0	0	0
2013	November	14	18	0	0	0	0	1	0

2013	November	14	19	0	0	0	0	0	0
2013	November	14	20	1	1	0	0	0	1
2013	December	2	1	0	0	2	1	0	3
2013	December	2	2	0	0	0	0	0	0
2013	December	2	3	0	1	0	0	0	0
2013	December	2	4	0	0	0	0	0	0
2013	December	2	5	0	0	1	0	0	0
2013	December	2	6	0	0	0	0	0	0
2013	December	2	7	0	0	0	0	1	0
2013	December	2	8	0	1	0	0	2	0
2013	December	2	9	0	0	0	0	0	1
2013	December	2	10	0	0	0	1	0	0
2013	December	2	11	0	0	0	0	1	0
2013	December	2	12	0	0	0	1	0	0
2013	December	2	13	0	0	1	1	0	0
2013	December	2	14	0	0	0	0	0	0
2013	December	2	15	0	0	2	0	0	0
2013	December	2	16	0	2	1	2	1	0
2013	December	2	17	0	0	0	1	1	1
2013	December	2	18	0	1	0	2	0	3
2013	December	2	19	0	0	1	0	1	2
2013	December	2	20	0	1	4	3	0	1
2013	December	3	1	0	0	0	0	1	1
2013	December	3	2	0	0	0	0	0	0
2013	December	3	3	0	0	0	0	0	1
2013	December	3	4	0	0	0	0	0	0
2013	December	3	5	0	0	0	1	0	0
2013	December	3	6	0	0	0	0	0	0
2013	December	3	7	0	0	0	0	0	0
2013	December	3	8	0	0	0	0	0	0
2013	December	3	9	0	0	1	0	0	0
2013	December	3	10	0	0	0	0	0	0
2013	December	3	11	0	0	0	0	0	0
2013	December	3	12	0	0	0	1	0	0
2013	December	3	13	0	0	0	0	0	0
2013	December	3	14	0	3	0	0	0	0
2013	December	3	15	0	0	1	0	0	0
2013	December	3	16	0	0	0	0	0	0
2013	December	3	17	0	0	0	0	0	0
2013	December	3	18	0	0	0	2	0	0
2013	December	3	19	0	0	0	0	1	0

2013	December	3	20	0	0	0	0	0	0
2013	December	6	1	0	3	0	0	0	0
2013	December	6	2	0	0	0	1	1	0
2013	December	6	3	0	0	0	0	0	3
2013	December	6	4	0	1	1	1	0	0
2013	December	6	5	0	0	0	0	0	0
2013	December	6	6	0	0	0	0	0	0
2013	December	6	7	0	0	1	0	0	0
2013	December	6	8	0	0	0	0	0	0
2013	December	6	9	0	0	1	2	0	0
2013	December	6	10	0	0	0	0	0	1
2013	December	6	11	0	0	0	0	0	0
2013	December	6	12	0	0	0	0	0	0
2013	December	6	13	0	0	0	1	0	0
2013	December	6	14	0	2	0	1	1	0
2013	December	6	15	0	0	1	0	2	0
2013	December	6	16	0	0	1	0	0	0
2013	December	6	17	0	1	0	0	0	0
2013	December	6	18	0	0	0	1	1	1
2013	December	6	19	0	1	0	0	0	0
2013	December	6	20	0	0	0	0	0	0
2014	January	12	1	0	0	0	0	0	0
2014	January	12	2	0	0	0	3	0	0
2014	January	12	3	0	0	1	0	1	0
2014	January	12	4	0	0	1	2	0	0
2014	January	12	5	0	0	1	0	0	0
2014	January	12	6	0	0	0	0	1	0
2014	January	12	7	0	0	2	1	1	0
2014	January	12	8	0	0	5	0	1	0
2014	January	12	9	0	0	0	1	0	0
2014	January	12	10	0	0	1	0	2	0
2014	January	12	11	0	0	0	3	1	0
2014	January	12	12	0	0	1	0	2	0
2014	January	12	13	0	0	1	0	0	0
2014	January	12	14	0	0	1	2	2	0
2014	January	12	15	0	0	1	0	4	0
2014	January	12	16	0	0	0	0	0	0
2014	January	12	17	0	0	2	2	2	0
2014	January	12	18	0	0	3	1	1	0
2014	January	12	19	0	0	1	0	0	0
2014	January	12	20	0	0	1	3	1	0

2014	January	14	1	0	0	0	0	1	0
2014	January	14	2	0	0	0	2	1	0
2014	January	14	3	0	0	0	1	0	0
2014	January	14	4	0	0	0	0	1	0
2014	January	14	5	0	0	0	2	0	0
2014	January	14	6	0	0	0	0	0	0
2014	January	14	7	0	0	2	5	0	0
2014	January	14	8	0	0	0	0	0	0
2014	January	14	9	0	0	0	2	1	0
2014	January	14	10	0	0	0	2	0	0
2014	January	14	11	0	0	1	1	1	0
2014	January	14	12	0	0	4	2	0	0
2014	January	14	13	0	0	0	0	2	0
2014	January	14	14	0	0	2	0	0	0
2014	January	14	15	0	0	1	0	1	0
2014	January	14	16	0	0	1	0	3	0
2014	January	14	17	0	0	0	0	0	0
2014	January	14	18	0	0	0	0	0	0
2014	January	14	19	0	0	1	0	0	0
2014	January	14	20	0	0	0	0	0	0
2014	January	11	1	0	0	0	2	0	0
2014	January	11	2	0	0	0	1	2	0
2014	January	11	3	0	0	2	1	1	0
2014	January	11	4	0	0	0	0	0	0
2014	January	11	5	0	0	0	0	3	0
2014	January	11	6	0	0	2	2	0	0
2014	January	11	7	0	0	0	1	1	0
2014	January	11	8	0	0	3	0	1	0
2014	January	11	9	0	0	0	1	1	0
2014	January	11	10	0	0	0	0	2	0
2014	January	11	11	0	0	1	2	0	0
2014	January	11	12	0	0	0	1	3	0
2014	January	11	13	0	0	1	0	1	0
2014	January	11	14	0	0	1	3	2	0
2014	January	11	15	0	0	0	0	0	0
2014	January	11	16	0	0	0	2	1	0
2014	January	11	17	0	0	2	0	0	0
2014	January	11	18	0	0	2	1	1	0
2014	January	11	19	0	0	0	0	0	0
2014	January	11	20	0	0	3	0	0	0
2014	February	11	1	0	0	0	2	0	0

2014	February	11	2	0	0	1	0	0	0
2014	February	11	3	0	0	0	0	0	0
2014	February	11	4	0	0	1	0	0	0
2014	February	11	5	0	0	0	0	0	0
2014	February	11	6	0	0	0	1	1	0
2014	February	11	7	0	0	0	1	0	0
2014	February	11	8	0	0	0	1	0	0
2014	February	11	9	0	0	1	0	2	0
2014	February	11	10	0	0	1	0	1	0
2014	February	11	11	0	0	0	0	0	0
2014	February	11	12	0	0	0	1	1	0
2014	February	11	13	0	0	1	0	2	0
2014	February	11	14	0	0	1	0	1	0
2014	February	11	15	0	0	1	0	0	0
2014	February	11	16	0	0	2	0	0	0
2014	February	11	17	0	0	0	0	0	2
2014	February	11	18	0	0	2	1	1	0
2014	February	11	19	0	0	0	0	0	0
2014	February	11	20	0	0	1	0	0	0
2014	February	3	1	0	0	0	2	0	0
2014	February	3	2	0	0	1	0	0	0
2014	February	3	3	0	0	0	0	0	0
2014	February	3	4	0	0	1	0	0	0
2014	February	3	5	0	0	0	0	0	0
2014	February	3	6	0	0	0	1	1	0
2014	February	3	7	0	0	0	1	0	0
2014	February	3	8	0	0	0	1	0	0
2014	February	3	9	0	0	1	0	2	0
2014	February	3	10	0	0	1	0	1	0
2014	February	3	11	0	0	0	0	0	0
2014	February	3	12	0	0	0	1	1	0
2014	February	3	13	0	0	1	0	2	0
2014	February	3	14	0	0	1	0	1	0
2014	February	3	15	0	0	1	0	0	0
2014	February	3	16	0	0	2	0	0	0
2014	February	3	17	0	0	0	0	0	2
2014	February	3	18	0	0	2	1	1	0
2014	February	3	19	0	0	0	0	0	0
2014	February	3	20	0	0	1	0	0	0
2014	February	2	1	0	0	2	3	2	0
2014	February	2	2	0	0	1	0	2	0

2014	February	2	3	0	2	0	0	1	0
2014	February	2	4	0	0	0	1	0	2
2014	February	2	5	0	0	0	0	0	0
2014	February	2	6	0	0	1	1	3	0
2014	February	2	7	0	1	0	2	0	0
2014	February	2	8	0	0	0	0	0	0
2014	February	2	9	0	0	0	0	0	0
2014	February	2	10	0	0	2	2	1	0
2014	February	2	11	0	1	0	0	0	0
2014	February	2	12	0	0	0	2	1	0
2014	February	2	13	0	0	2	1	2	0
2014	February	2	14	0	0	0	1	0	0
2014	February	2	15	0	0	1	0	2	0
2014	February	2	16	0	0	0	0	0	0
2014	February	2	17	0	0	0	1	0	0
2014	February	2	18	0	0	2	0	0	0
2014	February	2	19	0	0	0	1	1	0
2014	February	2	20	0	1	0	1	1	2
2014	February	12	1	0	0	1	2	1	0
2014	February	12	2	0	4	2	0	5	0
2014	February	12	3	0	3	1	1	1	0
2014	February	12	4	1	5	2	0	0	0
2014	February	12	5	0	1	3	2	1	0
2014	February	12	6	1	1	2	2	5	0
2014	February	12	7	0	4	2	5	3	0
2014	February	12	8	0	2	2	3	5	0
2014	February	12	9	0	0	2	2	3	0
2014	February	12	10	0	1	0	1	1	1
2014	February	12	11	0	4	4	1	0	0
2014	February	12	12	0	1	4	0	0	0
2014	February	12	13	0	2	4	6	1	0
2014	February	12	14	1	1	0	0	3	0
2014	February	12	15	0	0	2	0	2	0
2014	February	12	16	0	0	1	2	3	0
2014	February	12	17	0	0	1	1	4	0
2014	February	12	18	0	0	3	1	0	0
2014	February	12	19	0	0	5	0	2	0
2014	February	12	20	0	1	1	2	3	0
2014	March	3	1	0	0	0	0	0	0
2014	March	3	2	0	0	1	0	0	0
2014	March	3	3	0	0	1	0	1	0

2014	March	3	4	0	0	0	1	2	0
2014	March	3	5	1	0	1	2	0	3
2014	March	3	6	0	0	2	1	2	0
2014	March	3	7	0	0	2	1	2	0
2014	March	3	8	0	0	4	1	0	0
2014	March	3	9	0	0	2	1	1	0
2014	March	3	10	0	0	0	0	0	0
2014	March	3	11	1	0	4	2	0	0
2014	March	3	12	0	0	0	1	0	0
2014	March	3	13	0	0	2	1	1	0
2014	March	3	14	0	0	2	0	0	0
2014	March	3	15	0	0	0	0	0	1
2014	March	3	16	0	0	6	1	1	1
2014	March	3	17	0	0	0	0	0	0
2014	March	3	18	0	0	0	0	0	1
2014	March	3	19	1	0	2	1	0	1
2014	March	3	20	0	0	0	0	0	0
2014	March	11	1	0	0	0	0	0	0
2014	March	11	2	0	0	0	0	0	0
2014	March	11	3	0	0	0	0	0	0
2014	March	11	4	0	0	0	0	0	0
2014	March	11	5	0	0	0	0	0	0
2014	March	11	6	0	0	0	0	0	0
2014	March	11	7	0	0	0	0	0	0
2014	March	11	8	0	0	0	0	0	0
2014	March	11	9	0	0	0	1	0	0
2014	March	11	10	0	0	1	1	0	0
2014	March	11	11	0	0	0	0	0	0
2014	March	11	12	0	0	2	2	2	0
2014	March	11	13	0	0	0	0	0	0
2014	March	11	14	0	0	0	0	0	0
2014	March	11	15	0	0	0	1	0	0
2014	March	11	16	0	0	0	0	0	0
2014	March	11	17	0	0	0	0	0	0
2014	March	11	18	0	0	0	0	0	0
2014	March	11	19	0	0	0	0	0	0
2014	March	11	20	0	0	0	0	0	0
2014	March	13	1	0	0	2	1	0	0
2014	March	13	2	0	0	1	0	0	0
2014	March	13	3	0	1	0	0	0	0
2014	March	13	4	0	0	0	0	0	0

2014	March	13	5	0	0	0	1	0	0
2014	March	13	6	0	0	0	0	0	0
2014	March	13	7	0	0	0	0	0	0
2014	March	13	8	0	0	1	0	0	0
2014	March	13	9	0	0	0	1	0	0
2014	March	13	10	0	0	0	0	0	0
2014	March	13	11	0	0	0	0	0	0
2014	March	13	12	0	0	1	0	0	0
2014	March	13	13	0	0	0	1	0	0
2014	March	13	14	0	0	0	0	0	0
2014	March	13	15	0	0	0	0	0	0
2014	March	13	16	0	0	0	0	0	0
2014	March	13	17	0	0	0	0	0	0
2014	March	13	18	0	0	0	0	0	1
2014	March	13	19	0	1	0	0	0	0
2014	March	13	20	0	0	0	0	0	0
2014	March	14	1	0	0	0	0	0	0
2014	March	14	2	0	0	0	0	1	0
2014	March	14	3	0	0	0	0	0	0
2014	March	14	4	0	0	1	0	0	0
2014	March	14	5	0	0	1	0	1	0
2014	March	14	6	0	0	0	0	0	0
2014	March	14	7	0	0	0	0	0	0
2014	March	14	8	0	0	0	0	0	0
2014	March	14	9	0	0	0	0	0	0
2014	March	14	10	0	0	0	0	0	0
2014	March	14	11	0	0	0	0	0	0
2014	March	14	12	0	0	0	0	1	0
2014	March	14	13	0	0	0	0	0	0
2014	March	14	14	0	0	0	0	0	0
2014	March	14	15	0	0	0	0	0	0
2014	March	14	16	0	0	0	0	0	0
2014	March	14	17	0	0	0	0	0	0
2014	March	14	18	0	0	0	0	0	0
2014	March	14	19	0	0	1	0	0	0
2014	March	14	20	0	0	0	0	0	0
2014	March	9	1	0	0	0	0	0	0
2014	March	9	2	0	0	0	0	0	0
2014	March	9	3	0	0	0	0	0	0
2014	March	9	4	0	0	0	0	0	0
2014	March	9	5	0	0	0	0	0	0

2014	March	9	6	0	0	0	0	0	0
2014	March	9	7	0	0	0	0	0	0
2014	March	9	8	0	0	0	1	0	0
2014	March	9	9	0	0	0	0	0	0
2014	March	9	10	0	0	0	0	0	0
2014	March	9	11	0	0	0	0	0	0
2014	March	9	12	0	0	0	0	0	0
2014	March	9	13	0	0	1	0	0	0
2014	March	9	14	0	0	0	0	0	0
2014	March	9	15	0	0	0	0	0	0
2014	March	9	16	0	0	0	0	0	0
2014	March	9	17	0	0	0	0	0	0
2014	March	9	18	0	0	0	0	0	0
2014	March	9	19	0	0	0	0	0	0
2014	March	9	20	0	0	0	0	0	0
2014	March	10	1	0	0	1	0	0	0
2014	March	10	2	0	0	0	0	1	0
2014	March	10	3	0	0	0	0	0	0
2014	March	10	4	0	0	0	0	0	0
2014	March	10	5	0	0	0	0	0	0
2014	March	10	6	0	0	0	0	0	0
2014	March	10	7	0	0	0	0	0	0
2014	March	10	8	0	0	0	0	0	0
2014	March	10	9	0	0	0	0	0	0
2014	March	10	10	0	0	0	0	0	0
2014	March	10	11	0	0	0	0	0	0
2014	March	10	12	0	0	0	0	0	0
2014	March	10	13	0	0	0	0	0	0
2014	March	10	14	0	0	0	0	0	0
2014	March	10	15	0	0	1	0	0	0
2014	March	10	16	0	0	0	1	0	0
2014	March	10	17	0	0	0	0	0	0
2014	March	10	18	0	0	0	0	0	0
2014	March	10	19	0	0	0	0	0	0
2014	March	10	20	0	0	0	0	0	0
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2014	April	10	2	0	0	0	0	1	0
2014	April	10	3	0	0	0	0	0	0
2014	April	10	4	0	0	0	0	0	0
2014	April	10	5	0	0	0	0	0	0
2014	April	10	6	0	0	0	0	0	0

2014	April	10	7	0	0	0	0	0	0
2014	April	10	8	0	0	0	0	0	0
2014	April	10	9	0	0	1	0	0	0
2014	April	10	10	0	0	1	0	0	0
2014	April	10	11	0	0	0	0	0	0
2014	April	10	12	0	0	0	0	0	0
2014	April	10	13	0	0	0	0	0	0
2014	April	10	14	0	0	0	0	0	0
2014	April	10	15	0	0	0	0	0	0
2014	April	10	16	0	0	0	0	0	0
2014	April	10	17	0	0	0	0	0	0
2014	April	10	18	0	0	0	0	0	0
2014	April	10	19	0	0	0	0	0	0
2014	April	10	20	0	0	0	0	0	0
2014	April	4	1	0	0	0	0	0	0
2014	April	4	2	0	0	0	0	0	0
2014	April	4	3	0	0	0	0	0	0
2014	April	4	4	0	0	0	0	0	0
2014	April	4	5	0	0	0	0	0	0
2014	April	4	6	0	0	0	0	1	0
2014	April	4	7	0	0	0	0	0	0
2014	April	4	8	0	0	0	0	0	0
2014	April	4	9	0	0	1	1	0	0
2014	April	4	10	0	0	0	0	0	0
2014	April	4	11	0	0	0	0	0	0
2014	April	4	12	0	0	0	0	0	0
2014	April	4	13	0	0	0	0	0	0
2014	April	4	14	0	0	0	0	0	0
2014	April	4	15	0	0	0	0	0	0
2014	April	4	16	0	0	0	0	0	0
2014	April	4	17	0	0	0	0	0	0
2014	April	4	18	0	0	0	0	0	0
2014	April	4	19	0	0	0	0	1	0
2014	April	4	20	0	0	0	0	0	0
2014	April	9	1	0	0	0	0	0	0
2014	April	9	2	0	0	0	0	0	0
2014	April	9	3	0	0	0	0	0	0
2014	April	9	4	0	0	0	0	0	0
2014	April	9	5	0	0	0	0	0	0
2014	April	9	6	0	0	0	0	0	0
2014	April	9	7	0	0	0	0	0	0

2014	April	9	8	0	0	0	0	0	0
2014	April	9	9	0	0	2	0	0	0
2014	April	9	10	0	0	0	0	0	0
2014	April	9	11	0	0	0	0	0	0
2014	April	9	12	0	0	0	0	1	0
2014	April	9	13	0	0	0	0	0	0
2014	April	9	14	0	0	1	0	0	0
2014	April	9	15	0	0	0	0	0	0
2014	April	9	16	0	0	0	0	0	0
2014	April	9	17	0	0	0	0	0	0
2014	April	9	18	0	0	0	0	0	0
2014	April	9	19	0	0	0	0	0	0
2014	April	9	20	0	0	0	0	0	0
2014	April	9	1	0	0	0	0	0	0
2014	April	9	2	0	0	0	0	0	0
2014	April	9	3	0	0	0	0	0	0
2014	April	9	4	0	0	0	0	0	0
2014	April	9	5	0	0	0	0	0	0
2014	April	9	6	0	0	0	0	0	0
2014	April	9	7	0	0	0	0	0	0
2014	April	9	8	0	0	0	0	0	0
2014	April	9	9	0	0	0	0	0	0
2014	April	9	10	0	0	0	0	0	0
2014	April	9	11	0	0	0	0	0	0
2014	April	9	12	0	0	0	0	0	0
2014	April	9	13	0	0	0	0	0	0
2014	April	9	14	0	0	0	0	0	0
2014	April	9	15	0	0	0	0	0	0
2014	April	9	16	0	0	0	0	0	0
2014	April	9	17	0	0	0	0	0	0
2014	April	9	18	0	0	0	0	0	0
2014	April	9	19	0	0	0	0	0	0
2014	April	9	20	0	0	1	0	0	0
2014	April	12	1	0	0	0	0	0	0
2014	April	12	2	0	0	0	0	0	0
2014	April	12	3	0	0	0	0	0	1
2014	April	12	4	0	0	0	0	0	0
2014	April	12	5	0	0	0	0	0	0
2014	April	12	6	0	0	0	0	0	0
2014	April	12	7	0	1	0	0	0	0
2014	April	12	8	0	0	0	0	0	0

2014	April	12	9	0	0	0	0	0	1
2014	April	12	10	0	0	0	0	0	0
2014	April	12	11	0	0	0	0	0	0
2014	April	12	12	0	0	0	0	0	0
2014	April	12	13	0	0	0	0	0	0
2014	April	12	14	0	0	0	0	0	0
2014	April	12	15	0	0	0	0	0	0
2014	April	12	16	0	0	0	1	0	0
2014	April	12	17	0	0	0	0	0	0
2014	April	12	18	0	0	0	0	0	0
2014	April	12	19	0	0	0	0	0	0
2014	April	12	20	0	0	0	0	0	0
2014	April	14	1	0	0	0	0	0	0
2014	April	14	2	0	0	0	0	0	0
2014	April	14	3	0	0	0	0	0	0
2014	April	14	4	0	0	1	0	0	0
2014	April	14	5	0	0	0	0	0	0
2014	April	14	6	0	0	0	1	0	0
2014	April	14	7	0	0	0	0	1	0
2014	April	14	8	0	0	0	0	0	0
2014	April	14	9	0	0	0	0	0	0
2014	April	14	10	0	0	0	0	0	0
2014	April	14	11	0	0	0	0	0	0
2014	April	14	12	0	0	0	0	0	0
2014	April	14	13	0	1	0	0	0	0
2014	April	14	14	0	0	0	0	0	0
2014	April	14	15	0	0	0	0	0	0
2014	April	14	16	0	0	1	0	0	0
2014	April	14	17	0	0	0	0	0	1
2014	April	14	18	0	0	0	0	0	0
2014	April	14	19	0	0	0	0	0	0
2014	April	14	20	0	0	0	0	0	0