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Abstract approved:

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Janine E. Trempy

Cell-based biosensors are function-based detectors that use the physiological response of a living cell to sense biologically stimulating agents. This emerging technology extends the application of current detection methods by reporting on the toxicity of a sample and the potential to cause disease. Previously, *Betta splendens* erythrophores have been described as a method to detect toxic agents such as pesticides, chemicals, purified bacterial toxins and food-associated bacteria.

The first objective of this study was to examine the *B. splendens* erythrophore response to Gram-negative food-associated bacteria by investigating the response to *Salmonella typhimurium*. Erythrophores aggregated in the presence of *S. typhimurium* in a growth-phase-dependent manner and this response was distinct from the media control. Additionally, it was found that erythrophore aggregation was dependent on the bacterial cell and erythrophores were not responsive to secreted bacterial products found in the culture supernatant. Transposon mutagenesis of *S. typhimurium* ATCC 700720 resulted in an interruption of the promoter region of operon *prgHIJK*. This insertion inactivated the expression of the SPI-1 T3SS structural proteins. The mutation resulted in a delayed erythrophore aggregation, implying that the SPI-1 T3SS is an important component contributing to erythrophore aggregation.

The second objective of this study was to characterize the *Oncorhynchus tshawytscha* melanophore in terms of its responsiveness to aggregative and dispersive controls. It was hypothesized that the pigment response was biologically conserved between *O. tshawytscha* melanophores and *B. splendens* erythrophores. The data presented support this hypothesis and highlight potential applications. Melanophores

aggregated in response to mercuric chloride and sodium arsenite but dispersed in the presence of ammonia. Additionally, melanophores aggregated in response to salmonid bacterial pathogens *Aeromonas salmonicida*, *Yersinia ruckeri* and *Flavobacterium psychrophilum*. Melanophores were capable of differentiating between healthy and diseased fish tissues. These results warrant a continued investigation into the full potential of the *O. tshawytscha* melanophore system.

In conclusion, this study investigated two cell-based biosensor models; *B. splendens* erythrophores and *O. tshawytscha* melanophores. The data presented suggests that these models can be applied for the detection of foodborne bacterial pathogens and for the detection of chemical and bacterial contaminants pertinent to the aquaculture industry.

# Chromatophores as Cell-based Biosensors for the Detection of Chemically and Biologically Toxic Substances

by
Stephanie R. Dukovcic

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APPROVED:

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Major Professor, representing Microbiology

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Chair of the Department of Microbiology

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Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

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Stephanie R. Dukovcic, Author

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## CONTRIBUTION OF AUTHORS

Dr. Janine Trempy and Dr. Janine Hutchison were involved in the preparation of this dissertation. Dr. Janine Trempy, Dr. Janine Hutchison and Dr. Karen Dierksen were involved in organization and interpretation of this data. Dr. Janine Hutchison greatly contributed to the complementation assay depitcted in Figure 8 and Melissa Austin contributed the Clonidine and MSH data for *Betta splendens* erythrophores in Figures 9 and 10.

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For my sisters, Megan, Katie and Jenna

# Chromatophores as Cell-based Biosensors for the Detection of Chemically and Biologically Toxic Substances

## Chapter 1

## Introduction

Cell-based biosensors are viable eukaryotic cells that report the presence of toxicants of chemical or biological origin (19). These sensors possess an innate feature that can be exploited to detect toxic substances and much research has gone into optimizing cell-based biosensors for food- and water-based analyses. Chromatophores have emerged as an intriguing class of cell-based biosensors that respond to contaminants based on a function dependent mechanism.

Chromatophores are a class of brilliantly colored pigment cells found in fish, amphibian, and cephalopod species (99). Chromatophores are subdivided according to the color of their intracellular pigment organelles; erythrophores (red), melanophores (brown to black), xanthophores (yellow), cyanophores (blue), leucophores (white) and iridophores (metallic or iridescent) (107). Chromatophores are capable of regulating the location of their pigment organelles in response to extracellular stimuli. Aggregation describes pigment movement towards the central perinuclear region, and conversely, dispersion indicates pigment movement towards the outer periphery of the chromatophore. Changes in pigment distribution can be rapid and reversible and confer camouflage abilities for many species. Pigment movement also reflects environmental imbalances as well as the physiological stress of a species (99). This innate ability of chromatophores to move their pigment organelles in response to extracellular stimuli makes them an excellent cell-based biosensor model to study.

Erythrophores isolated from *Betta splendens* have been described as cell-based biosensors for the detection of toxic agents such as pesticides, chemicals, purified bacterial toxins, and more recently, Gram-positive foodborne bacteria (71, 134, 194). The evaluation of *B. splendens* erythrophores in response to Gram-negative foodborne bacteria has not been well described.

The objectives of this study are twofold: (1) to further expand our knowledge of *B. splendens* erythrophores for the detection of Gram-negative foodborne bacterial pathogen *Salmonella typhimurium*, and (2) to introduce for the first time, the use of melanophores from *Oncorhynchus tshawytscha* (Chinook salmon) as cell-based biosensors.

The first focus of this study is to describe the interaction between *B. splendens* erythrophores and *S. typhimurium*. Previous work has shown that erythrophores respond to *Salmonella enteritidis* (134); although the mechanisms underlying the interaction between erythrophores and *Salmonella* spp. is not well understood. Furthermore, the erythrophore response to *S. typhimurium* has not been explored prior to this body of work. The data presented supports the hypothesis that *B. splendens* erythrophores respond to *S. typhimurium* by aggregating their intracellular pigment organelles, similar to the erythrophore response to *S. enteritidis*. This body of work provides evidence that *B. splendens* erythrophores can be used as cell-based biosensors for the detection of *S. typhimurium*, and the molecular mechanisms behind this interaction was investigated.

The second focus of this study is to characterize *O. tshawytscha* melanophores as a cell-based biosensor. Melanophores have been subjected to aggregative and dispersive criteria previously established for *B. splendens* erythrophores (71, 194). The evidence presented here defends the hypothesis that pigment movement in response to explicit stimuli is conserved between chromatophores of *O. tshawytscha* and *B. splendens* in primary cell culture. This study shows promise for melanophores to be used for the evaluation of water contaminants and salmonid bacterial pathogens, both in culture and from diseased fish tissues.

I propose that the continued investigation of *B. splendens* erythrophores, *O. tshawytscha* melanophores and chromatophores from uninvestigated species to act as cell-based biosensors for the detection of chemical and biological contaminants is warranted, as chromatophores from one species may be more pertinent for the analysis of a particular contaminant over another species' chromatophores. The data presented here will highlight interesting qualities of each model system, while

suggesting future directions that will undoubtedly enhance the biosensing capabilities of both erythrophores and melanophores.

# Chapter 2
# Literature Review

This chapter will be broken into two parts. The first will introduce bacterial foodborne illness, with a focus on current detection methods and will highlight emerging biosensor technologies as they pertain to the food industry. Emphasis will be placed on *Salmonella* associated foodborne illness, and a current understanding of *Salmonella* pathogenesis will be discussed. The second part of this chapter will include a discussion on water-based contamination and bacterial pathogens that afflict salmon species, as each of these areas were investigated as potential applications for *Oncorhynchus tshawytscha* melanophores.

## Part I: *Salmonella* Associated Foodborne Illness and Detection

### *Foodborne Illness*

Microorganisms are ubiquitous, thriving in many diverse environments including taking up residence in food and water. While many microorganisms do not pose a threat, and some even supplement dietary needs, some can become pathogenic or act as opportunistic pathogens that can cause gastrointestinal infections upon consumption of contaminated food. While most infections are self-limiting and do not require hospitalization, serious complications can arise especially for infants, the elderly and immunocompromised individuals, and may even result in death.

Among the most prevalent bacterial culprits of foodborne illness are *Campylobacter* spp., *Salmonella* spp., *Clostridium perfringens*, *Staphylococcus* spp., *Escherichia coli* and *Listeria monocytogenes,* in order from most to least prevalent (187). *Salmonella* spp. are the second most prevalent gastrointestinal bacterial infection and are the leading cause of death due to foodborne illness in the USA, and globally, are responsible for over 1.3 billion cases of gastrointestinal disease each year (55, 146, 187, 269). *Salmonella* outbreaks are classically linked to contaminated meat and dairy products, although outbreaks have also been associated with tomatoes, orange juice, sprouts, spinach and peanuts in recent years. Economic loss associated with *Salmonella* infections, including estimated medical costs and loss of

productivity, is estimated to exceed several billion dollars annually in the USA alone (93). The incredible impact that *Salmonella* has in terms of health risk and economic loss warrants the need for improved detection mechanisms and preventative measures.

Concerns of bacterial pathogens being used as bioweapons and the risk of intentional tampering of the food supply has highlighted the need to develop rapid, sensitive and specific methods of pathogen detection. Of additional concern are newly emerging or unknown bacterial pathogens that often evade detection using traditional detection methods. Ideally, novel methods would be implemented as preventative measures and identify contamination problems before the product reaches the consumer.

*Foodborne Pathogen Detection*

Methods of foodborne pathogen detection have evolved considerably over the last century. Traditional techniques include enrichment, culturing bacteria on artificial media, and classification based on bacterial metabolism. Within the food industry, food samples must be processed, which often includes extraction, purification, enrichment and separation steps. These methods can be laborious, expensive and time consuming, and thus have largely been replaced by immunological and molecular techniques.

Immunological or antibody-based tests such as the enzyme linked immunosorbent assay (ELISA) are among the most widely used to determine unknown bacterial contaminants in food. This assay detects the presence of a conserved protein structure. Primary polyclonal or monoclonal antibodies are used to target an array of related structures or a specific structure, respectively. Secondary enzyme-labeled antibodies are used to target the primary, and then detected visually or by a spectrophotometer following the addition of a chromogen. Many different ELISA assays exist, including those that employ the use of antibody coated magnetic beads or nanoparticles to confer greater specificity and sensitivity (109). Some ELISA protocols are coupled with a polymerase chain reaction (PCR) protocol in an effort to be even more selective for particular organisms.

PCR techniques are based on the detection of a nucleic acid, either DNA or RNA, and have evolved tremendously since PCRs original debut some thirty years ago (199). PCR involves the use of primers, template (DNA or RNA), dNTPs and a thermostable polymerase. Through a series of denaturing, annealing and elongation steps, a target sequence is amplified exponentially and subsequently viewed after gel electrophoresis. Many different PCR protocols exist today that are widely implemented for the detection of foodborne pathogens. Reverse transcriptase PCR (RT-PCR) uses mRNA for template, and therefore distinguishes viable from non-viable organisms. Multiplex PCR is particularly useful as it allows for the simultaneous detection of several organisms by utilizing multiple primer pairs for the same template. Real-time PCR has been developed to obtain rapid results by quantifying fluorescent emissions with each amplification cycle, thus eliminating analysis by gel electrophoresis. More recently, microarrays have been developed to offer high throughput nucleic acid detection. While not a PCR protocol, microarrays use hundreds to thousands of nucleic acid probes that can be designed to target many sequences relevant to pathogenic foodborne bacteria. Despite the convenience of high throughput analysis, microarrays can be expensive and require a great deal of preparatory work and analysis. Even with the development of rapid and sensitive PCR protocols, false positives and negatives remain a predicament and sample preparations can still be time consuming.

Both ELISA and PCR based methods target a known structure and are therefore biased towards the detection of a particular organism and are limited in their ability to detect unknown or emerging pathogens. Further, these assays do not address toxicity, the ability to cause disease, or the viability of a particular organism (except RT-PCR), contributing to false positives (68, 256). Sample processing is particularly crucial to the accuracy of these assays. The presence of carbohydrates, lipids and proteins in high levels will interfere with both ELISA and PCR. Toxicity assays are often used in tandem or alone in order to overcome some of these problems.

Toxicity assays, using live animal models can assess if a sample is toxic or has the potential to cause disease. Examples of animal models include canaries that have been used to monitor carbon monoxide levels in coal mines. Mice are routinely used

to study microbial pathogenesis, although disease progression does not always correlate to a human response from the same pathogen. Transgenic fish are commonly used as toxicity sentinels for a number of chemical and biological contaminants in aquatic environments (275). While live animals do address the potential of an organism to cause disease, they require a great deal of care and maintenance and they are not practical as portable sensing assays. Detection mechanisms that can match the toxicity of whole animal models and the specificity of molecular/immunological techniques as well as providing a rapid yet accurate result are the cornerstones for developing any new method. Biosensors are an emerging technology that offer specificity and sensitivity by exploiting physiological reactions, and are therefore able to distinguish between viable and nonviable samples.

**Biosensors**

Biosensors are biological entities that respond to biological and chemical stimulants in a physiological manner that can be quantified. The fundamental aspect of biosensors is their ability to physiologically report the presence of a contaminant based on function, rather than structure. As a result, biosensors address the toxicity of viable organisms and/or their associated toxins, thus overcoming the limitations of immunological and molecular based assays. Biosensor design varies greatly but all models posses a biological sensing component (antibody, enzyme, nucleic acid, receptor, organelle, prokaryotic cell, eukaryotic cell, etc.) and a transducer or signaling component based on fluorescence, optics, mass, electrochemical gradients or otherwise physiological activity (19, 168, 172, 217). Biosensors have shown great promise with applications towards clinical diagnoses, environmental monitoring and food analysis (90, 137, 172). While sample preparation remains an obstacle for many biosensor devices, some are tolerant to different food matrices making them very appealing for use in monitoring and detecting food samples (217).

Many biosensors have already been described for the detection of foodborne pathogens and include but are not limited to: surface plasmon resonance (SPR), resonant crystal, chemiluminometric and fluorescently-labeled biosensors (172, 217).

An intriguing and newer branch of biosensor technology are those that utilize an entire cell, either prokaryotic or eukaryotic, as their primary sensing device.

**Cell-based Biosensors**

Cell-based biosensors are isolated cells, and are emerging as biological toxicity sensors for the functional detection of toxic agents such as chemicals, pathogenic bacteria and their associated toxins. As opposed to whole animal toxicity assays, isolated cells can be analyzed without the cross-talk interference of differing cell types in the whole organism, and therefore simplify the analysis while retaining specificity (19). Cell-based biosensors have the potential to transform monitoring, screening, and detection of biological and chemical toxicants for a number of industries including food, environmental, pharmaceutical, military and national security.

Cell-based biosensors can be categorized into two general groups: (i) those that have been modified for detection; engineered cell-based biosensors, or (ii) those that relay a measurable signal through inherent physiological properties; innate cell-based biosensors. Engineered cell-based biosensors offer a high level of specificity, and two prominent examples for bacterial foodborne detection are the rat basophilic leukemia mast cell and the B-lymphocyte cell. Rat basophilic leukemia mast cells injected with fluorescent indicator dyes release fluorescent exocytotic granules in response to alkalinization, calcium fluxes or the presence of reactive oxygen species (63). The mast cell biosensor was further engineered to express an IgE chimeric protein receptor as an indicator for *Escherichia coli* and *Listeria monocytogenes*. Similarly, by measuring alkaline phosphatase release, B-lymphocyte cells immobilized in a collagen matrix have been engineered to respond to *L. monocytogenes*, purified listeriolysin O toxin and purified *Bacillus* enterotoxin, all the while distinguishing these responses from nonpathogenic *Listeria innocua* and *Bacillus subtilis* (20). Engineered cell-based biosensors are undoubtedly specific, but represent years of culminating evidence from inherent cell physiology and natural receptor-ligand interactions.

Innate cell-based biosensors rely on natural receptor binding events and intracellular signal transduction pathways to convey a measurable signal. Chromatophore-based biosensors are particularly interesting because they are innate biosensors and are analytically easy to interpret. Chromatophores are a class of pigment cells that are subdivided according to the color of their intracellular pigment organelles: erythrophores (red), melanophores (brown), cyanophores (blue), xanthophores (yellow), leucophores (white) and iridophores (iridescent). Chromatophores are present in a number of amphibian and fish species and are physiologically important for camouflage and communicating during mating rituals. Fish are known to alter their appearance in response to environmental cues and stress as well as harmful and infectious agents by either aggregating or dispersing their intracellular pigment organelles and respectively appearing lighter or darker in overall color (171, 272, 276, 279). Pigment aggregation occurs in response to G-protein coupled receptor binding events that stimulate dynein, a microtubule associated motor protein in eukaryotes, which translates to the movement of pigment organelles towards the microtubule organizing center and surrounding the nucleus (228). Increased levels of $Ca^{2+}$ and decreased levels of cAMP are associated with pigment aggregation (14, 254). Conversely, opposing ligands can translate to pigment dispersion, in which case the microtubule motor protein kinesin would be stimulated to transport pigment organelles to the periphery of the cell (228). Pigment dispersion is dependent on $Ca^{2+}$ and associated with increased cAMP levels (136, 213). Pigment movement in cell culture, either aggregation or dispersion, can be easily monitored by capturing time lapsed images and measuring changes in optical density.

Examples of chromatophore innate cell-based biosensors include *Xenopus laevis* (frog) melanophores and *Betta splendens* (Siamese fighting fish) erythrophores. *Xenopus laevis* melanophores, while not an example of foodborne pathogen detection, have been shown to respond to an array of hazardous water contaminants (264). This *X. laevis* melanophore biosensor has since been engineered for the specific detection of opiates in blood plasma, urine and saliva (145). Similar to melanophores, erythrophores have recently been described for their potential as a cell-based biosensor. Erythrophores from *B. splendens* exhibit varying intracellular pigment

aggregation rates for the detection of *Bacillus cereus*, *Clostridium perfringens*, *C. botulinum* and *Salmonella enteritidis*, and importantly, do not elicit a response when exposed to nonpathogenic *B. subtilis* or non-pathogenic *B. cereus* mutants (133, 134). *Betta splendens* erythrophores have also been used to detect various chemical agents linked to water contamination and purified bacterial toxins (71, 194). Erythrophores show incredible promise as sensors capable of detecting toxic conditions, and given the described sensitivity for foodborne pathogens, may also respond to additional pathogens.

To better understand the potential of erythrophore cell-based biosensors, significant research into the molecular and cellular interactions involved is critical. *Salmonella typhimurium* was selected as the model pathogen for this study in order to better understand erythrophore-bacterial interactions. The next portion of this review will focus on the current knowledge of *Salmonella* pathogenesis, shedding light on potential host cell-pathogen interactions and resulting signal transduction pathways to exploit for biosensing capabilities.

*Salmonella enterica*

The *Salmonella* genus belongs to the Gram-negative *Enterobacteriaceae* family and consists of two species, *Salmonella enterica* and *Salmonella bongori*. Each species is classified into subspecies and even further divided into serotypes. *Salmonella* typing has evolved from the conventional species-specific serological classification based on O (somatic) and H (flagellar) antigens, and is now configured using a serotype-specific antigenic scheme developed by the World Health Organization Collaborating Centre for Reference and Research on *Salmonella* (WHO Collaborating Centre) (35). *Salmonella enterica* subsp. *enterica*, to date, has the most known human pathogenic serotypes. These medically relevant serotypes are commonly referred to in an abbreviated form; *Salmonella typhi*, *S. paratyphi*, *S. typhimurium* and *S. enteritidis* to name a few. While *S. typhi* and *S. paratyphi* are exclusively maintained within human reservoirs, *S. typhimurium* and *S. enteritidis* are capable of colonizing a wide array of animals and humans. Most *Salmonella* infections result from the ingestion of contaminated food or water, and in healthy

individuals the infection is self-limiting. Common disease manifestations among all *Salmonella* serovars include nausea, vomiting, diarrhea, fever and abdominal cramps. The typhoidal serovars, *S. typhi* and *S. paratyphi* may develop septicemia and become further complicated by typhoid fever which occurs when the bacteria disseminate and replicate in the liver, spleen, bone marrow and gallbladder. It is estimated that over 20 million cases of enteric (typhoid) fever occur annually due to typhoidal *Salmonella* serovars (62). Non-typhoidal serovars such as *S. typhimurium* and *S. enteritidis* can also result in systemic infections and can induce chronic conditions such as reactive arthritis, an autoimmune disease also known as Reiter's syndrome (196).

*Salmonella* disease results from an infectious dose that can vary from $10^1$ to $10^{11}$ CFU depending on the serovar and the food it occupies (196). Upon ingestion of contaminated food, the bacteria survive the acidic stomach environment and invade the small intestine. *Salmonella* will enter specialized microfold cells (M cells); non-ciliated, non-mucus producing epithelial cells located within Peyer's patches. Bacterial invasion is mediated by the *Salmonella* pathogenicity island 1 (SPI-1) type III secretion system (T3SS) and intracellular survival and replication is orchestrated by the SPI-2 T3SS.

Most *Salmonella* infections in healthy people result in an immune response that successfully clears the infection. When intestinal epithelia are infiltrated by *S. typhimurium*, the chemokine interleukin (IL)-8 is released in order to recruit neutrophils to the site of infection (184, 185). Neutrophils release cytotoxic granules to induce mucosal sloughing which effectively clears *Salmonella* in the form of diarrhea.

*Salmonella Pathogenesis*

While many *Salmonella* species share similar pathogenic strategies, the focus of this literature review will be on *Salmonella enterica* subsp. *enterica* serovar Typhimurium, which will be referred to as *S. typhimurium* from this point forward.

The *S. typhimurium* route of infection begins with the oral ingestion of contaminated food or water. Following the digestive tract, *S. typhimurium* enters the stomach and withstands the gastric pH by expressing acid shock and outer membrane

proteins that are part of the acid tolerance response (ATR). The ATR response can be induced either through pH-dependent processes or by the nutrient deprivation alternative sigma factor, $\sigma^s$ (196). Once within the small intestine, *S. typhimurium* employs an arsenal of virulence factors that protect the bacterium from digestive enzymes, bile salts, secretory IgA, antimicrobial peptides and additional immune defenses as *S. typhimurium* traverses the intestinal mucosa and invades the underlying intestinal epithelium.

*Salmonella typhimurium* utilizes fimbrial adhesins to stick to the intestinal epithelium which aggravates the brush border and contributes to inflammatory diarrhea. *Salmonella typhimurium* preferentially invades M cells in the distal small intestine (Figure 1) (142, 154). M cells are interspersed among ciliated epithelial cells and are associated with Peyer's patches, the epicenter of immunological recognition in the small intestine. M cells do not secrete mucus and are therefore devoid of IgA, allowing pathogens to exploit M cells as a gateway to the underlying tissues. *Salmonella typhimurium* enters M cells by bacterial-mediated endocytosis through the deployment of the SPI-1 T3SS and the release of effector proteins that specialize in actin cytoskeleton rearrangement and promotes bacterial uptake (50, 100). Bacterial mediated endocytosis results in the formation of the *Salmonella*-containing vacuole (SCV), an endosome that *S. typhimurium* occupies and permits bacterial replication. *Salmonella typhimurium* intracellular survival is dependent upon the SPI-2 T3SS which injects effector proteins across the SCV membrane and into the cytoplasm, providing various evasion strategies but ultimately provides a mechanism for the bacteria to escape lysosomal fusion (39, 75). The SCV can associate with the M cell phospholipid membrane at the basal surface, thus releasing *S. typhimurium* into the extracellular space. Macrophages reside on the basal side of M cells within Peyer's patches, and phagocytoze *S. typhimurium*. Macrophages can mount an immune response to effectively clear a *S. typhimurium* infection but particularly in immunocompromised individuals, the SPI-2 T3SS can permit *S. typhimurium* survival within the macrophage which can lead to a systemic infection.

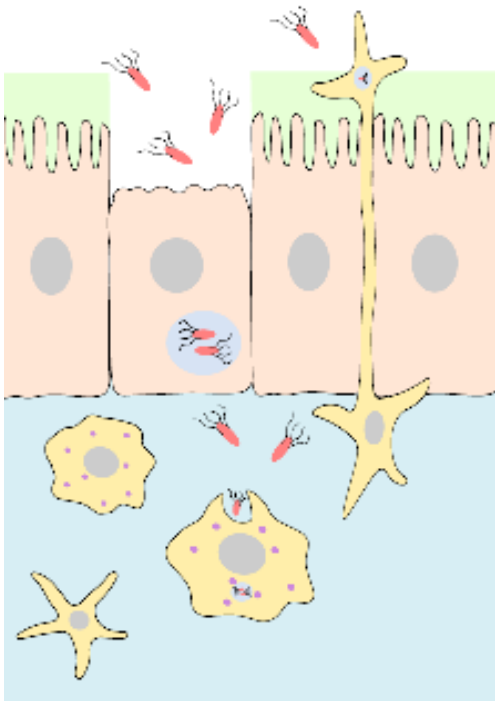

**Figure 1**: *Salmonella* invasion of the intestinal epithelia. *Salmonella* preferentially invade microfold cells (M cells), which are non-ciliated epithelial cells that do not secrete mucus. M cells are located between ciliated epithelial cells in regions called Peyer's patches, which are concentrated immunological centers. Macrophages engulf *Salmonella* that enter the extracellular space following M cell transcytosis to the basal surface. Dendritic cells sample the intestinal lumen and engulf *Salmonella*.

Additionally, *S. typhimurium* can cross the intestinal epithelial barrier by hitching a ride with CD18+ myeloid dendritic cells (267). Dendritic cells residing on the basal side of the intestinal epithelium project dendritic arms between epithelial cells to sample the intestinal lumen, and as a consequence, engulf invading pathogens. While this mechanism of *Salmonella* invasion and its importance towards *Salmonella* persistence remains unclear, it may represent a strategy for dissemination (116).

The ability of *S. typhimurium* to invade and survive inside M cells, macrophages and dendritic cells is due to the expression of virulence factors. In *Salmonella*, these factors are often organized in specific genomic regions.

*Salmonella Pathogenicity Islands*

The *Salmonella* genome contains horizontally acquired clusters of chromosomally encoded virulence factors called *Salmonella* pathogenicity islands (SPIs). At present, twelve SPIs have been described (120), with some SPIs specific to particular serovars while others are common to all *S. enterica* serovars. The following represents a brief overview of SPIs and their major characteristics.

SPI-1 is a 40 kb island conserved among all *Salmonella* species and contains genes important for iron uptake as well as bacterial invasion, including structural proteins for a T3SS, its associated effector proteins that are secreted into the host cell and key regulators (100). SPI-2 is 40 kb, specific to *S. enterica* serovars. This pathogenicity island harbors genes important for intracellular survival (238), including a second T3SS responsible for secreting effector proteins into the host cell cytoplasm from its intracellular replicating niche; the SCV. SPI-3 is a 17 kb locus that encodes a high-affinity $Mg^{2+}$ uptake system, crucial for adaptation to the nutrient poor SCV environment, thus promoting *Salmonella* intracellular survival (27, 28). SPI-4 is 27 kb and encodes a putative type I secretion system (T1SS), although many of its genes remain uncharacterized (277). SPI-5 is a relatively small locus of 7.6 kb and contains genes *sopB* and *pipB*, effectors that are translocated by the SPI-1 and SPI-2 T3SSs respectively (120). A locus of 59 kb is called SPI-6 for *S. typhi* and the same locus is referred to as *Salmonella* chromosomal island (SCI) in *S. typhimurium*, which seems to contribute at least in part, to bacterial invasion (91, 208). SPIs with alternative names include the *Salmonella* genomic island 1 (SGI-1) and the high pathogenicity island (HPI). SGI-1 is 43 kb and has been linked to several multidrug resistant strains of *Salmonella*, contributing to tetracycline, ampicillin, chloramphenicol, streptomycin and sulfonamide resistance (30). HPI is 35-45 kb depending on the serotype, and was initially characterized in *Yersinia* spp. (237). HPI, present in several *Salmonella* serovars but absent from human-specific serovars, encodes genes important for iron acquisition (205).

*Salmonella typhimurium* does not possess SPIs 7, 8, 9 or 10 (120, 183). Interestingly, the 133 kb SPI-7 locus, also known as major pathogenicity island (MPI), encodes the SPI-1 T3SS effector protein SopE (113, 283). SopE is a phage

encoded gene and is present in *S. typhimurium* (192, 193), which suggests horizontal transfer at different evolutionary times.

This represents a general description of virulence gene organization. The following provides a more detailed analysis of the roles of *S. typhimurium* virulence factors that contribute to pathogenicity.

*Salmonella Virulence Factors*

Virulence factors are bacterial products that contribute to some manifestation of disease either directly or indirectly. Mechanisms that are associated with bacterial virulence include adherence, invasion, toxin production/secretion, capsule formation, immune evasion strategies, resistance to antibiotics/antimicrobials and regulation of virulence gene expression. Known *S. typhimurium* virulence factors are extensive, this review will focus on factors leading to or directly involved in invasion.

Adhesins can be classified into two distinct groups based on their structure and function; fimbrial and non-fimbrial (242). Fimbrial protein adhesins, or pili, are heteropolymers anchored to the outer membrane and associate with host cell receptors to facilitate cell to cell contact and relay pertinent signals. Non-fimbrial protein adhesins function in the same general capacity but are composed of a single protein or homo-oligimer. Each type of adhesin is also capable of adhering to abiotic surfaces and many are implicated in biofilm formation. Outer membrane adhesion composition within a single species may vary according to diverse environmental conditions or to confer specificity to different hosts and tissues (104). Known *Salmonella* fimbrial adhesions include RatB, SinH and type I pili Fim, Lpf and Pef. Non-fimbrial *Salmonella* adhesions include MisL, ShdA and SiiE. Adhesions with known contributions to biofilm formation include Agr or curli (fimbrial) and BapA (non-fimbrial). Adhesins and their contributions to disease are reviewed by Gerlach and Hensel 2007 (104).

The key to mounting a pathogenic attack is the controlled expression of virulence factors, appropriately timed to reflect environmental changes, allowing bacteria to adapt to and survive the diverse surroundings they encounter. *Salmonella* possess a sophisticated web of interconnected global and gene-specific regulators responsible

for activating or repressing genes contributing to virulence at the transcriptional and translational levels. Perhaps the best studied global regulator in *Salmonella* is the PhoP/PhoQ two component regulatory system. The PhoPQ system is activated by a mildly acidic pH, antimicrobial peptides and low $Mg^{2+}$ levels consistent with the intracellular environment, suggesting that the PhoPQ system is important for regulating gene expression to accommodate intracellular survival (214). Also promoting intracellular survival and replication are regulators CRP and SpvR, as well as alternative sigma factors RpoS, RpoH and RpoE, which are activated when *Salmonella* encounters nutrient deprivation and enters starvation mode (50, 54, 135, 257).

Additional two component systems and their contributions to virulence regulation include: the PreA/PreB role in motility and quorum sensing (188, 189), the RcsC/YojN/RcsB system effect on capsule synthesis and motility (198), the OmpR/EnvZ and SsrB/SsrA systems and their regulation of SPI-1 and SPI-2 genes (21, 53, 80, 86), and SirA/BarA regulation of *scrB* and *hilD* expression which ultimately affects SPI-1 genes (11, 82, 165).

Arguably, the most significant contributions to virulence are the *Salmonella* type III secretion systems encoded by SPI-1 and SPI-2, which are discussed in detail below. In terms of regulation, HilA and InvF are the major players and directly bind to promoters in SPI-1. HilA binding activates the *prg/org*, *inv/spa* and *sic/sip* operons, while InvF requires the help of SicA to activate SPI-1 genes (67, 78). HilC, HilD and RtsA are responsible for positively regulating HilA, although are also capable of binding to the key SPI-1 promoters independently of HilA (67, 78, 81, 177, 206, 233). Even Fur, the ferric uptake regulator has a role in SPI-1 activation and is thought to act upon HilA indirectly and likely through HilD (83). HilE negatively regulates SPI-1 genes by binding and inactivating HilD (25). All the while, many of these regulators are themselves, regulated by the global systems mentioned above, but most pathways seem to incorporate HilD in some capacity.

**Gram-negative Secretion Systems**

Gram-negative bacteria have evolved a number of secretion pathways to transport proteins to their outer membrane surface, extracellular space, or directly into the cytoplasm of a host cell. Several of these systems are fairly sophisticated and involve complex macromolecular structures to circumvent the limitations of having an outer membrane, as opposed to Gram-positive bacteria. To date, nine secretion systems have been identified in Gram-negative bacteria that can be divided into two major classes: (i) sec-dependent pathways; and, (ii) sec-independent pathways.

Sec-dependent pathways include the type II secretion system (T2SS); the type V secretion system (T5SS); the chaperone/usher (CU) system and the extracellular nucleation-precipitation (ENP) system. In short, T2SSs transport hydrolytic enzymes and toxins to the extracellular environment and type IV pili to the outer membrane (211, 230, 244). The T5SS is clever in its simplicity; substrates mediate their own transport across the outer membrane after being targeted through inner transmembrane protein complexes (104, 156). The CU system is best understood for its transport of type I pili in *Escherichia coli*. The CU system involves a periplasmic chaperone, FimC and an usher, FimD that creates an assembly foundation on the outer membrane (232, 268). Finally, the ENP system is important for biofilm production and transports adhesive fibers called curli to the outer membrane (176, 244).

Sec-independent pathways include the type I secretion system (T1SS); type III secretion system (T3SS); the type IV secretion system (T4SS) and the twin-arginine translocation (Tat) system. A newly discovered secretion system, type VI (T6SS) has similarities to the T4SS but is still poorly understood. Notably, the T4SS does exist as a sec-dependent transport pathway for a few documented organisms (47). Briefly, T1SSs transport a variety of high molecular weight substrates with a C-terminal signal sequence directly into the extracellular space without cleaving the signal. The α-hemolysin (HlyA) of pathogenic *Escherichia coli* is the best described T1SS exotoxin (155). T4SSs are evolutionary related to conjugation machinery and have the unique ability to transport proteins and single-stranded-DNA-protein complexes in either a cell contact-dependent or –independent manner (47, 72, 175). The Tat

system is known to translocate proteins in their quaternary structure that possess an N-terminal peptide signal containing two arginines, using the proton-motive force as its driving power (231). T3SSs, particularly as they pertain to *Salmonella* species, will be discussed in greater detail throughout the remainder of this literature review.

**Type III Secretion Systems**

Type III secretion systems (T3SSs) are evolutionarily related to flagellar export systems and are distinguishable by their signature needle-like apparatus that protrudes from the bacterial outer membrane. This secretion apparatus is composed of more than 20 proteins and can be divided into the following substructures: base, inner rod, needle and translocon. The multi-ring base spans the inner and outer bacterial membranes and serves as an anchor for the inner rod which also associates with the hollow needle (151, 160). The translocon proteins are secreted and assemble in the host cell membrane to create a pore and target for the needle apparatus (150). Structural components of the T3SS are relatively conserved between species, although the cocktail of effector proteins released by T3SSs appear to be unique among different species. *Salmonella* possess two T3SSs that are assembled and activated at different times during the infection (112). The SPI-1 encoded T3SS is critical for bacterial invasion while the SPI-2 encoded T3SS is imperative for intracellular replication and survival. *Salmonella* invade non-phagocytic cells, such as M cells, by deploying the SPI-1 T3SS, inducing bacterial-mediated endocytosis, and gaining access to the host cell intracellular environment. The resulting endocytozed vesicle is called the *Salmonella*-containing vacuole (SCV) and serves as a protective replicative niche provided that the SPI-2 T3SS and its arsenal of effector proteins are able to neutralize the host cells defense mechanisms.

To date, the characterization of the SPI-1 T3SS far outweighs the characterization of the SPI-2 T3SS. As a result, the SPI-1 T3SS will be discussed in detail, and when appropriate, known details pertaining to the SPI-2 T3SS will be shared.

**Salmonella SPI-1 T3SS Apparatus**

The *S. typhimurium* SPI-1 T3SS base structure is formed by equimolar amounts of three sec-dependent proteins: InvG, PrgH and PrgK (Figure 2). InvG forms in the outer most membrane and PrgH and PrgK associate with each other in the inner most cytoplasmic membrane. The export machinery that resides within the PrgH/PrgK inner membrane ring is composed of several proteins: InvC, InvA, SpaP, SpaQ, SpaR and SpaS. InvC is an ATPase and recognizes the subsequent structural T3SS proteins and unfolds them to facilitate efficient migration through the base and subsequent formation of the inner rod and needle, formed by PrgJ and PrgI respectively.

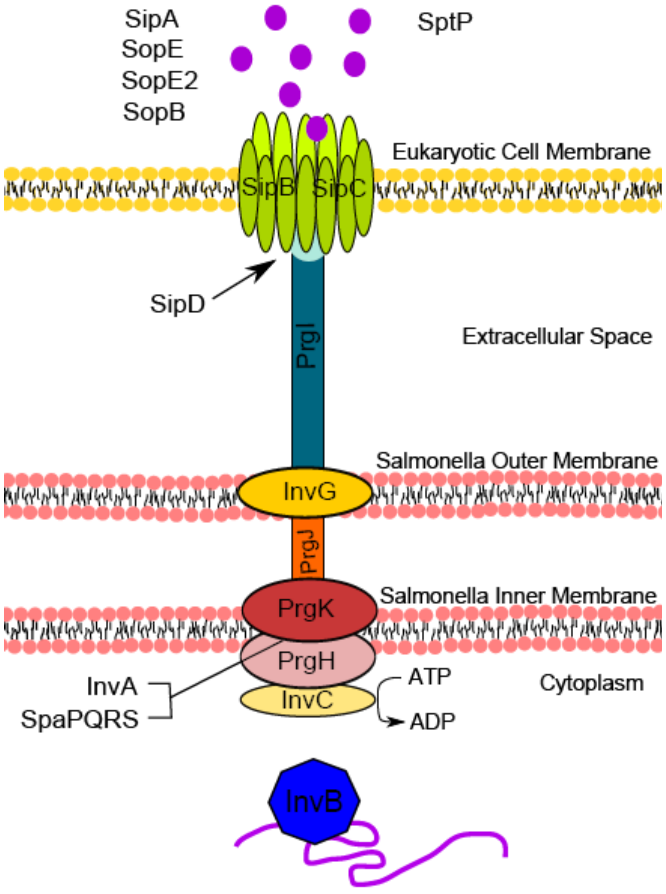


**Figure 2**: *Salmonella* SPI-1 T3SS apparatus and translocon structure. PrgH, PrgK and InvG form the primary base structural components. PrgJ forms the inner rod and PrgI forms the needle-like apparatus. SipD associates with the tip of the needle, and together with SipB and SipC, forms the translocon in the host cell membrane. See text for details.

Various chaperones and stabilizing proteins are involved throughout the assembly process. InvJ ensures proper needle complex assembly yet does not remain as a permanent structural protein (159, 179). Likewise, InvH escorts InvG to form the outer most ring in the base structure although it does not participate in the final T3SS structure (61, 66). IagB is proposed to play a role in joining the InvG structure with the PrgH/PrgK structure and facilitating safe passage through the peptidoglycan layer (100, 281). The multicomponent export machinery is conditionally specific in what proteins it allows to pass through the needle complex, a process referred to as substrate switching. InvJ seems to play a major role in substrate switching, contributing to conformational changes in the secretion apparatus that switch from being competent for PrgJ and PrgI passage to competency for effector protein export (57). Effector proteins are escorted to the T3SS apparatus by various chaperones, including InvB, which is specific towards effectors SipA, SopE and SopE2 (36, 77). These underlying mechanisms are still poorly understood, although evidence supports a model for substrate-specificity switching that occurs after the completion of inner rod formation (178).

The translocon pore is composed of SipB, SipC and SipD, which are secreted through the T3SS apparatus and associate with the host cell phospholipid bilayer (56). SipD is presumed to be located at the tip of the needle complex, just as its *Shigella* homolog IpaD (84). Recent evidence shows that SipD associates with the host cell membrane immediately upon contact and initiates translocon assembly (163). SipB and SipC were not detected immediately upon host cell contact (163), yet are critical for a properly functioning translocon and likely associate soon after SipD deployment. Interestingly, the translocon location within the host membrane does not appear to be random; rather, SipB binds cholesterol rich microdomains called lipid rafts with high affinity (118). SipB may either target lipid raft regions, or on the contrary, may recruit cholesterol mobilization to the site of translocon assembly. Lipid rafts are implicated as highly desirable portals for the T3SSs of many species including but not limited to *S. typhimurium*, *Shigella flexneri*, *Pseudomonas aeruginosa* and *Yersinia enterocolitica* (103, 118, 127, 161, 236).

**SPI-1 T3SS Effectors**

*S. typhimurium* bacterial-mediated endocytosis is orchestrated by the SPI-1 T3SS effector proteins. In particular, SipA (also called SspA) is an effector that directly binds to actin and induces polymerization while enhancing the activity of SipC (also called SspC), which nucleates and bundles actin (119, 234, 285). SipC is also a major component of the translocon, but has a cytoplasmic domain capable of binding to actin filaments in the host cell. Aside from directly binding actin, SopE and SopE2 induce actin cytoskeletal rearrangements and membrane ruffling by mimicking host cell guanine-nucleotide exchange factors (GEFs) and stimulating host Rho GTPases (210). SopE activates host cell proteins Rac-1 and Cdc42, while SopE2 preferentially stimulates Cdc42; each catalyzing the conversion of bound GDP (inactive state) to GTP (active state). These phosphorylated Rho GTPases become activated and stimulate downstream pathways through Arp2/3 which leads to assembly, branching and polymerization of new actin filaments (18, 95, 106, 117, 209, 247, 284). SopB contributes to cytoskeletal rearrangements through inositol phosphatase activity, targeting host GEFs to activate the GTPase RhoG (209, 210). The described activities of SipA, SipC, SopE, SopE2 and SopB, in concert, promote bacterial uptake via macropinocytosis. SopB and SopD then cooperate with each other to rapidly seal membrane invaginations and form stable SCVs (17, 210). As if to leave no trace behind, *Salmonella* encode the effector SptP to return the host cytoskeleton to its pre-engulfment state by inactivating Cdc42 and Rac-1 (210). SptP possesses two known catalytic domains; an N-terminal RhoGAP domain and a C-terminal tyrosine phosphatase domain (144). GAPs, or GTPase-activating proteins, antagonize the activity of GEFs by stimulating the hydrolysis of bound GTP to its inactive GDP form and thereby terminating the signal of that particular GTPase. Therefore, the RhoGAP activity of SptP acts to counter the GEF activities of SopE and SopE2 (96, 97, 245). The tyrosine phosphatase activity of SptP targets tyrosine kinase ACK, a downstream effector of Cdc42, and vimentin, an intermediate filament protein, in order to promote cytoskeletal recovery following *Salmonella* internalization (200). Interestingly, SptP, SopE and SopE2 are injected simultaneously in comparable molar amounts. SopE and SopE2 are degraded quickly whereas SptP has a much longer half

life, providing temporal regulation of actin cytoskeletal rearrangement followed by cytoskeletal recovery (158). Despite efforts to cover tracks and evade the host immune system, several SPI-1 T3SS effector proteins stimulate a proinflammatory response during the invasion phase.

**SPI-1 T3SS Effectors and Inflammation**

SPI-1 T3SS effectors invariably stimulate host defense mechanisms which clinically present as acute intestinal inflammation. When Cdc42 is activated by SopE, SopE2 and SopB, not only does it lead to cytoskeletal rearrangement, but also to Raf1-dependent upregulation of Erk, Jnk and p38 mitogen-activated protein kinase (MAPK) pathways and subsequent activation of AP-1 and NF-κB transcription factors (167, 209, 210). As a consequence, the proinflammatory cytokine IL-8 (also known as CXCL8) is released by the invaded epithelial cells, and is a major chemoattractant for neutrophils to the site of infection. Also contributing to neutrophil recruitment, hepoxillin A3 is released following a SipA triggered Arf6- and phospholipase D signaling cascade (167, 271). Neutrophil transmigration across the epithelial monolayer is encouraged by the activities of SopB, SopE, SopE2 and SipA, which have been shown to disrupt tight junctions; thus providing an avenue for neutrophils (31). Transmigration, along with chloride secretion and fluid flux from infected epithelia (stimulated by SopB and SipA), contributes to diarrhea (31, 167, 210). While diarrhea is an effective elimination of enteric pathogens in healthy individuals, it also promotes bacterial transmission to other hosts via the fecal-oral route.

*Salmonella* are capable of invading and replicating within epithelial cells, dendritic cells and macrophages. Throughout the infection process, host cells undergo programmed cell death in an effort to eliminate the pathogen. Interestingly, depending on cell type and the stage of infection, one of three SPI-1 dependent pathways towards programmed cell death can occur in response to invading *Salmonella*. One, infected intestinal epithelial cells exhibit the classic apoptotic pathway through activation of caspase-3 and caspase-8 (207, 282), which appears to be dependent on bacterial invasion, intracellular replication and the release of SPI-2

T3SS effectors (149, 207). The particular effectors responsible for activating caspase-3 and caspase-8 remain elusive. Two, infected macrophages and dendritic cells experience caspase-1 mediated pyroptosis (124, 266), which is dependent upon SPI-1 T3SS effectors and flagellin (92, 190). SPI-1 T3SS components and effectors that contribute to pyroptosis include InvA, InvG, InvJ, PrgH, SipB, SipC, SipD and SpaO but SipA and SptP have been eliminated as potential contributors (34, 51, 124, 140, 195, 265). Caspase-1 mediated pyroptosis, unlike other forms of programmed cell death, facilitates an inflammatory response through the release of cytokines IL-1β and IL-18 upon cell lysis, which results in fever and recruitment of additional immune cells (32). Three and finally, infected macrophages have been observed to experience caspase-1 independent cell death in caspase-$1^{-/-}$ mice, which requires the SPI-1 T3SS, and only SipB has been eliminated as a potential effector involved in this pathway (123, 140). This pathway has been described as apoptosis (140) and autophagy (123) but a conclusive verdict has not been reached. Each of these mechanisms for *Salmonella* induced programmed cell death serves as an attempt to protect the host and prevent the spread of infection by either eliciting an inflammatory response, in the case of pyroptosis, or the compartmentalized lysis and subsequent phagocytosis of compromised cells in the case of apoptosis and autophagy.

**SPI-1 T3SS Effectors and Evasion**

Additional SPI-1 T3SS effectors are responsible for intercepting host cell signal transduction pathways to prevent immunological recognition. Initial studies have indicated that the SPI-1 T3SS effector AvrA is linked to proinflammatory apoptosis (58), yet recent evidence has surfaced to suggest that AvrA works to repress the apoptotic defense mechanism (143). The difference in results has been attributed to using cell culture and nonpathogenic *Salmonella* strains (58) versus whole organism models and pathogenic *Salmonella* strains (143). Abiding by the later study, AvrA appears to promote *Salmonella* survival and evasion of the innate immune response by inhibiting JNK-mediated apoptosis through its acetyltransferase activity targeting the MAPK pathway (143). In general, the MAPKs function as a phosphorylation relay team to activate transcription factors that allow transcription of pertinent genes

that convey immunological signals. The MAPK pathway is a three tiered cascade; the MAP kinase kinase kinases (MAPKKKs) phosphorylate the MAPKKs which subsequently phosphorylate the MAPKs and in turn activate transcription factors. AvrA specifically blocks the MAPKK MEKK4/7 (143), which normally functions to phosphorylate c-Jun N-terminal kinase (JNK), whose downstream substrate is Jun. Together, Jun and Fos (activated through a different pathway) form the transcription factor AP-1 (139). This particular MAPK pathway leads to proinflammatory gene expression, and during prolonged activation, leads to the induction of proapoptotic pathways (274). Therefore, AvrA functions to suppress the innate immune response. To that effect, AvrA has also been shown to inhibit neutrophil transmigration by stabilizing tight junctions (173).

The multifunctional AvrA protein also inhibits the inflammatory response by obstructing NF-κB signaling, blocking IL-8 production and preventing ubiquitination of β-catenin (58, 251).Ubiquitination involves three enzymes, E1, E2 and E3 which bind to proteins and target them to the proteosome for degradation. β-catenin has a short half-life and a high turnover rate. When β-catenin ubiquitination is blocked, β-catenin accumulates in the nucleus and binds to the transcription T cell factor (TCF) which leads to the activation of several genes, some of which are linked to cell proliferation (8, 212, 278). Maintaining β-catenin homeostasis is critical, and an imbalance of synthesis and degradation has been linked to tumorigenesis (152, 197, 212, 278). β-catenin normally functions in association with α-catenin to connect cadherins and actin filaments, forming adheren junctions between adjacent epithelial cells. These structures result in a continuous actin belt that spans the epithelial layer.

Modulating the ubiquitination activies of the host cells seems to be a reoccurring theme with SPI-1 T3SS effectors. SlrP is a putative ubiquitin ligase with yet to be determined host cell targets, but along with effector SspH1, is known to confer virulence in claves and may grant host specificity (263). SspH1 is a known E3 ubiquitin ligase that targets PKN1, inhibiting NF-κB dependent gene expression and therefore repressing proinflammatory cytokine production (114, 115, 222). Interestingly, SlrP and SspH1 along with effectors SteA and SteB (unknown functions) can be secreted by both the SPI-1 and SPI-2 secretion apparatus (191).

*Research Objectives*

The first focus of this study is to describe the interaction between *B. splendens* erythrophores and *S. typhimurium*. Previous work has shown that erythrophores respond to *Salmonella enteritidis* (134); although, the mechanisms underlying the interaction between erythrophores and *Salmonella* spp. is not understood. Further, the erythrophore response to *S. typhimurium* has not been explored prior to this body of work.

It was hypothesized that *B. splendens* erythrophores aggregate in the presence of *S. typhimurium*, similar to the response observed with *S. enteritidis*. To investigate this hypothesis, the following questions were asked: (i) do *B. splendens* erythrophores respond to *S. typhimurium*, (ii) if so, can this response be optimized, and (iii) what bacterial products are involved in stimulating the erythrophore response? The data that follows in the subsequent chapters addresses these questions. This body of work provides evidence that *B. splendens* erythrophores can be used as cell-based biosensors for the detection of *S. typhimurium*.

Part II: Melanophores and Water-based Contamination

The second part of this chapter introduces melanophore biology of teleosts (bony fish) and provides a review on water based contamination in terms of chemical pollution and bacterial pathogens that afflict salmon species, with an emphasis on conditions relevant to the Pacific Northwest region.

*Melanophore Biology*

Melanophores are a chromatophore cell type with brown to black pigment, as discussed earlier in this chapter. Like erythrophores, melanophores are capable of distributing their intracellular pigment organelles in response to external stimuli. Pigment movement towards the center of the cell describes pigment aggregation, and this movement concentrates the color of the cell around the nucleus, making the cell appear smaller. Conversely, pigment movement to the outer periphery of the cell is pigment dispersion and reveals the dendritic morphology of melanophores. Rapid relocation of pigment organelles occurs naturally in response to neurotransmitter and

hormonal release and is physiologically important for camouflage and communicating during mating rituals (126, 174, 270). In addition to these innate responses, fish are known to alter their appearance in reaction to stress as well as harmful and infectious agents by either aggregating or dispersing their intracellular pigment organelles (171, 272, 276, 279).

As discussed earlier in this chapter, pigment movement is microtubule associated and occurs in response to G- protein coupled receptor binding events. Increased intracellular $Ca^{2+}$ levels and decreased cAMP translate to pigment aggregation involving the motor protein dynein (14, 228, 254). Kinesins are responsible for pigment dispersion which is linked to increased intracellular cAMP levels and is dependent upon $Ca^{2+}$ (136, 213, 228). Early studies using fish melanophores has shown that removal of microtubules does not completely inhibit pigment movement (235). Since this discovery, actin filaments located in the cell periphery and myosin motor proteins have been implicated in both aggregation and dispersion. This movement involves pigment organelles switching tracks between microtubules and actin filaments (221). Organelle transport involving the different cytoskeletal components, and switching between these tracks is a relatively new observation, but it has been hypothesized that pigment organelles bind multiple motor proteins simultaneously (13).

Melanophore pigment organelles are composed of melanin and have been shown to bind a wide variety of toxic substances. Melanin acts as an antioxidant and is capable of binding superoxide radicals, singlet oxygen and hydroxyl radicals (225), and therefore likely serves to protect melanophores from reactive oxygen species. Additionally, melanin is capable of binding cationic metals such as calcium, iron, copper and magnesium (162, 255). Melanin can sequester drugs and chemicals such as organic amines and polycyclic aromatic hydrocarbons and store them within melanophores (164). Chronic exposure to particular drugs and chemicals can exhaust melanin binding activity resulting in the release of these toxic substances into the intracellular or extracellular space, which is contingent upon the ability of the particular toxin to cause melanophore lysis. Given the described toxin binding

abilities of melanin, *Oncorhynchus tshawytscha* (Chinook salmon) melanophores were tested for their ability to respond to water contaminants.

*Water Contamination and the Aquaculture Industry*

Anthropogenic pollution describes substances that persist in the environment as a result of human activities, and are generally toxic to human and animal health. Waterways naturally contain chemical substances in trace amounts, but anthropogenic contributions such as industrial effluents, agricultural runoff (pesticides, fertilizers, enteric pathogens), sewage treatment plants and storm water overflow account for ecological imbalances of chemical substances. Fish species in contact with abnormal substances in the water column can become chemical reservoirs; most notably, mercury bioaccumulates in marine life to toxic levels, affecting fish and human health (253). Additional chemicals of concern as the result of anthropogenic pollution include ammonia, copper, arsenic and cyanide.

Concern over human health with exposure to abnormal levels of water contaminants demands research into the effects of these toxic substances on fish health. The average individual in the United States consumes over 16 lbs of fish each year (6). The Oregon commercial fishing industry contributed in excess of $400 million in personal income to the economy in 2006 (108), the most recent year for which statistics are available. On the national scale, commercial fishing provided 1.5 billion jobs and corresponded to over $40 billion in personal income (1). The Oregon Department of Fish and Wildlife (ODFW) operates over 30 hatchery facilities with the intent of conserving native fish populations while supporting sport, commercial and tribal fishing. Water quality is of utmost importance to hatcheries and precautions such as feeding regimens, settling tanks and clarifiers are used to minimize adverse ecological impacts to the surrounding watersheds. Chemicals of particular importance that warrant effluent monitoring in hatcheries include phosphorous and nitrogenous compounds as well as solid waste from excess feed (3). Of additional importance, is monitoring fish health. Oregon hatcheries commonly use hydrogen peroxide or formalin as antifungal agents as well as florfenicol or oxytetracycline to help control

bacterial agents such as *Aeromonas salmonicida*, *Flavobacterium columnare* and *Flavobacterium psychrophilum*.

Detection of bacterial pathogens in hatchery reared fish is often accomplished through gross pathological examination, bacterial culture and histological analysis, although typical bacterial problems are often recognized based on seasonal occurrence and observations based on previous outbreaks. Often, bacterial problems are recognized when a mass mortality among the population of fish occurs. Diagnosis has improved with the development of immunological assays and molecular techniques, but these can be costly procedures and samples are often outsourced as this equipment may not be readily available at fish hatchery sites. Phenotypic tests involving the use of selective or differential media are more practical for hatchery use. Melanophores represent a novel technology for the detection of bacterial pathogens and would supplement techniques already in use.

*Salmonid Bacterial Pathogens*

Fish have been observed to alter their appearance in response to harmful and infectious agents (171, 272, 276, 279); therefore, *O. tshawytscha* melanophores have been tested for their ability to respond to salmonid bacterial pathogens *Aeromonas salmonicida*, *Yersinia ruckeri*, *Flavobacterium psychrophilum* and *Carnobacterium piscicola*. The following represents a brief communication on these bacterial pathogens that primarily infect salmonid species.

***Aeromonas salmonicida***

*Aeromonas salmonicida*, a Gram-negative bacterial pathogen, is classically viewed as the causative agent of furunculosis, an often lethal disease that manifests as musculature lesions protruding through the dermis (16). The bacterium has a fairly broad host range and is capable of disseminating and causing systemic disease. Geographically, *A. salmonicida* has been isolated from wild and farmed fish worldwide. The first report of furunculosis in North America was in 1902 and the causative agent was initially described as *Bacillus truttae* (180). Despite being recognized as a salmonid pathogen for over a century, the route of infection remains

ambiguous and controversy persists concerning the ability of *A. salmonicida* to exist as a free-living organism independent of the fish host (16). Contributing to the difficulty of isolating *A. salmonicida* from the aquatic environment, the bacterium is capable of entering a viable but non-culturable state (9). Asymptomatic carriers account for a substantial source of infection for healthy fish, as do deceased fish infected with *A. salmonicida*. Vertical transmission has been investigated in several studies but seems to be inconclusive at this time (16). Known virulence factors include the S-layer and its associated adhesins (261), siderophores FstC, FstB and HupA (76), superoxide dismutase SodA and SodB (22, 64, 102), glyerophospholipid:cholesterol acetyltransferase (GCAT) and the serine protease AspA (229). Additionally, recent studies have identified a T3SS in *A. salmonicida* (42, 43, 65).

***Yersinia ruckeri***

The host range for Gram-negative *Y. ruckeri*, the causative agent of enteric redmouth disease (ERM), is primarily restricted to salmonid species, and particularly *Oncorhynchus mykiss* (Rainbow trout) (40). *Yersinia ruckeri* commonly affects fish approximately 7.5 cm in length and results in a less severe but chronic infection in larger fish. Stressed fish, as may be the case in crowded tanks, are more susceptible to *Y. ruckeri* infection (132, 226). Just as the name implies, the classic symptom of ERM is a reddening of the mouth and throat caused by subcutaneous hemorrhaging (45). Bacteremia can occur, which allows the bacteria to disseminate to the kidney and spleen, and melanosis has also been observed in *Y. ruckeri* infections (98). It has been suggested that asymptomatic carriers may occur due to the cyclic nature of *Y. ruckeri* outbreaks (46). The general consensus among researchers is that *Y. ruckeri* is capable of surviving outside of the host in the water and mud sediments following an outbreak for as long as 2 months (16, 153, 186). Alternative reservoirs for the pathogen include crayfish, muskrats, and potentially, ornamental species such as goldfish (182, 248). Geographically, *Y. ruckeri* is largely isolated to the western hemisphere, and has continued to be a problem for Pacific Northwest fish hatcheries (125). Like *A. salmonicida*, *Y. ruckeri* can also exist in a viable but non-culturable state (223), which

has complicated laboratory analysis. Known *Y. ruckeri* virulence factors include biofilm formation (60), extracellular protease Yrp1 (87) and hemolysin YhlB (89) as well as siderophores RucC and RupD (88). A T3SS has also been recently identified in *Y. ruckeri* and contributes to the pathogenicity of this organism (110).

***Flavobacterium psychrophilum***

*Flavobacterium psychrophilum* is a Gram-negative bacterial pathogen and the causative agent of bacterial coldwater disease (BCWD); characterized by lesions on the fins and saddle-like lesions within the dermis near the dorsal fin. The reservoir harboring *F. psychrophilum* is undetermined but some evidence exists to support transovarian transmission through salmonid eggs (37). It has been observed that fish infected with *F. psychrophilum* appear darker in color, indicating an increase in the number of chromatophores in the dermis and/or pigment dispersion (171, 276, 279). Complete genome sequencing has revealed many putative virulence factors, although only a few have been characterized. Known pathogenic mechanisms include gliding motility encoded by *tlpB* (12) and biofilm formation (74), although even these virulence factors are poorly understood. Significantly more research has been conducted on *Flavobacterium columnare*, which can induce the formation of yellow to orange lesions on the gills, fins or skin. *Flavobacterium columnare* and *F. psychrophilum* may share additional characteristics, such as constituting the normal gill microflora of healthy salmonids (260), and occurring naturally in a free-living form in the aquatic environment (227).

***Carnobacterium piscicola***

*Carnobacterium piscicola* is a Gram-positive pathogen that can cause varying disease symptoms including internal hemorrhaging, abdominal distension, fluid accumulation, muscle abscesses and septicemia (16). Disease often occurs in post-spawning fish and can be easily misinterpreted as stress responses, although generally it is not associated with fish mortality. Loss does occur on a few occasions and treatments must be performed in order to control outbreaks. It is unclear if *C. piscicola* is a natural aquatic microflora or if fish are the natural host. Nevertheless,

*C. piscicola* seems to be confined to North American and European waters. Studies concerning *C. piscicola* pathogenicity are limited but it has been shown that exotoxins do not contribute to the adverse effects of *C. piscicola* induced disease (59, 130, 224), the potential role of endotoxins has yet to be ascertained.

*Research Objectives*

The second focus of this study is to characterize *O. tshawytscha* melanophores as a cell-based biosensor. Melanophores have been subjected to aggregative and dispersive criteria previously established for *B. splendens* erythrophores (71, 194).

It was hypothesized that the pigment response was biologically conserved between chromatophores of *O. tshawytscha* and *B. splendens* in primary cell culture. To test this hypothesis and provide evidence for the development of an *O. tshawytscha* melanophore cell-based biosensor, the following questions were asked: (i) is the response conserved, and (ii) if so, can the *O. tshawytscha* melanophore be used to detect water contaminants and salmonid pathogens pertinent to the aquaculture industry? This study shows promise for melanophores to be used for the evaluation of water contaminants and salmonid bacterial pathogens, both in culture and from diseased fish tissues.

# Chapter 3
# Materials and Methods

## Primary Cell Culture

### *Erythrophore Isolation from Betta splendens*

Red male *Betta splendens* were used exclusively and purchased from either local pet stores or aquariamfish.net. Fish were maintained individually at 28°C for one month prior to erythrophore extraction, with alternating dark and light 12 hour cycles and fed every other day. Once a week their water was changed and the fish were dipped in a 3% aquarium salt solution. These husbandry conditions were optimized to decrease fungal, parasitic and bacterial infections and were implemented according to the regulations of Oregon State University's Institutional Animal Care and Use Committee (approval #2979 and #3513). Fish were anesthetized in an ice water bath for approximately 20 minutes prior to the erythrophore extraction procedure. Erythrophores were extracted from the caudal, dorsal and anal fins as previously described (194).

Briefly, each erythrophore preparation consisted of cells harvested from a single fish. Fin tissue was clipped and briefly washed in 25 mL of phosphate buffer saline [PBS, 128 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, 1.46 mM $KH_2PO_4$, supplemented with 5.6 mM glucose and 1% antibiotic/antimycotic (Gibco #20012043), pH 7.3]. Fins were transferred to a 15 mL Falcon tube with 10 mL skinning solution [SK, 128 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, 1.46 mM $KH_2PO_4$, 1 mM EDTA, supplemented with 5.6 mM glucose and 1% antibiotic/antimycotic (Gibco #20012043), pH 7.3]. Fins were washed for 1 minute in SK on a rocker plate, SK was removed by pipetting and 10 mL of fresh SK was added. Fins received 10 SK wash steps in total. This step was critical for removing scales and non-pigmented epithelial cells from the fin tissue.

Erythrophores were released from the fin tissue following a wash step in a digestive enzyme solution [ENZ, 20-30 mg collagenase type I (Worthington #LS004196), 1-3 mg hyaluronidase (Worthington #LS002594), 7 mL PBS, 0.2 μm filter sterilized]. Fins were exposed to ENZ by rocking for approximately 1-10

minutes. Length of exposure varied between erythrophore preparations. Erythrophores were separated from remaining fin tissue by transferring the ENZ to a new 15 mL Falcon tube and centrifuging for 3 minutes at 600 rpm using a swinging bucket centrifuge (IEC Centra CL3). The ENZ supernatant was decanted back onto the fin tissue for the subsequent erythrophore digestion, and the above digestion and separation steps were repeated as needed. The erythrophore cell pellet was resuspended in 100-500 µL Leibovitz L15 medium [L15+ (Gibco #21083-027) supplemented with 2% 1 M HEPES (Gibco #15630-080) and 1% antibiotic/antimycotic (Gibco #20012043)], depending on the size of the erythrophore pellet obtained. Pellets from subsequent centrifugations were kept separate and plated on either a 24-well or 48-well microtiter plate.

Erythrophores were plated by pipetting a 5 µL drop into the center of a well. Cells were allowed to attach to the surface for 15-20 minutes. L15+ medium was then added to the cells to a final volume of 1.5 mL (24-well) or 750 µL (48-well) and allowed to equilibrate for 20 minutes. Finally, 5% fetal bovine serum (FBS, HyClone #SH30071-01) was added to each well. Plates were kept at room temperature, and media was changed weekly to remove cellular waste products until the cells were no longer physiologically responsive.

*Melanophore Isolation from Oncorhynchus tshawytscha*

The Oregon Department of Fish and Wildlife (ODFW) generously provided hatchery raised *Oncorhynchus tshawytscha* (Chinook salmon) from various Oregon hatcheries, from June through February, for all melanophore preparations and analyses. Melanophores were extracted from the dorsal and tail fins of young salmon 6 to 20 cm in length. Melanophore cell culture preparation was conducted very similar to the erythrophore preparation described above with only a few modifications. To compensate for a smaller melanophore yield per salmon, the fins of 3 to 4 salmon were combined into a single melanophore preparation. Additionally, fin tissue was soaked in PBS for 30 minutes and the SK solution wash time was extended to 8-10 minutes per wash. Finally, L15+ was also supplemented with 2% Fungizone® (Invitrogen, #15290-018) an antimycotic.

Response Assays and Computational Analysis

Erythrophores and melanophores (collectively termed chromatophores) were tested for their physiological responsiveness with the addition of clonidine hydrochloride [to induce pigment aggregation, (Sigma-Aldrich, #C-7897)] or α-melanocyte stimulating hormone [MSH, to induce pigment dispersion (Sigma-Aldrich, #M4135)] at a final concentration of 100 nM prior to each experiment. Once cells were determined to be viable and physiologically responsive, they were used in various biological and chemical assays. For each assay, a population of chromatophores viewed at 40X were observed through a Leica (Leica, Inc., Wetzlar, Germany) DMIL inverted microscope (Bartels and Stout) with a mounted SPOT Insight 320 color camera (Diagnostic Instruments, Inc., Sterling Heights, MI, USA). Chromatophores were exposed to biological and chemical agents (diluted 1:10 upon addition to a well) and changes in pigment area occupied by the population of cells were recorded. This was accomplished by capturing time lapsed images using SPOT Advanced software version 3.5.6.2 (Diagnostic Instruments, Inc.). Experiments with erythrophores were analyzed using Image Pro Plus 4.1 (Media Cybernetics, Bethesda, MD, USA) and Excel. Area was calculated in pixels and the change in pigment area was determined using the following equation:

$$\text{Change in Pigment Area} = - [(A_o - A_t)/A_o] \times 100$$

where $A_o$ is the initial area occupied at time zero, and $A_t$ is the area at any time t. Data was represented graphically by plotting "change in pigment area" versus time. An aggregative response correlates to a negative percent area change whereas a dispersive response is indicative of a positive percent area change. Each erythrophore assay was conducted at least twice.

The computational analysis for melanophores was optimized to account for a lower cell density. The entire population of erythrophores within in the field of vision was included in the percent area change calculation. On the other hand, melanophore change in pigment area was calculated for individual cells, typically 3 to 4 cells per analysis, and averaged together to represent the total response. This discrepancy was

critical to eliminate the influence of background debris (fungal or algal) on altering melanophore changes in pigment area as well as offering a more accurate analysis that accommodates the lower cell density. Data from individual melanophores that skewed dramatically from the overall response were dropped from the computational analysis.

Melanophore Longevity Study

To ascertain how long melanophores remain viable in primary cell culture, three melanophore preparations were examined over 6 weeks. Following cell culture preparations, melanophores were allowed to equilibrate for 3 days. Week 1 data was collected on day 4 and subsequent weekly analyses were conducted 7 days apart. Cell culture media was changed weekly and melanophores were given 3 days to equilibrate before experimental analysis. Melanophores were exposed to 100 nM clonidine and observed for a total of 10 minutes by taking time lapse images. No data was collected during week 6 because all three melanophore preparations were fully aggregated and no longer physiologically responsive.

Melanophore Neurotransmitter Analysis

Melanophore response to aggregative and dispersive agents has not been well understood. In order to characterize the *O. tshawytscha* melanophore, and highlight its potential to be used as a cell-based biosensor, melanophores were exposed to neurotransmitters adenosine hemisulfate salt, clonidine hydrochloride, dopamine hydrochloride, serotonin hydrochloride and the adenyl cylcase activator MSH at effective concentrations previously reported for *B. splendens* erythrophores (Table 1) (71). Each was purchased through Sigma-Aldrich, prepared as 4X solutions and diluted to their final concentrations upon addition to a well of melanophores. Time lapse images were captured and analyzed as described above and all experiments were conducted in triplicate.

**Table 1**: Neurotransmitter and MSH concentrations. Agents were tested at the concentrations indicated on *O. tshawytscha* melanophores. MSH (α-melanocyte stimulating hormone). All agents were purchased through Sigma-Aldrich.

| Agent | Catalog Number | Concentrations Tested |
|---|---|---|
| Adenosine | A-7636 | 100 nM, 1 µM, 10µM, 100 µM, 1 mM, 10 mM |
| Clonidine | C-7897 | 1 nM, 10 nM, 100 nM, 1 µM, 10µM |
| Dopamine | H-8502 | 100 pM, 1 nM, 10 nM, 100 nM, 1 µM, 10µM, 100 µM, 1 mM |
| Serotonin | H-9523 | 1 µM, 10µM, 100 µM, 1 mM, 10 mM |
| MSH | M-4135 | 1 pM, 10 pM, 100 pM, 1 nM, 10 nM, 100 nM, 1 µM |

Melanophore Responsiveness to Water Contaminants

Melanophores were exposed to the following chemicals at concentrations relevant to human health: the military exposure guideline (MEG) and the human lethal concentration (HLC), representing a sublethal and lethal concentration respectively (Table 2) (264) The following chemicals were prepared as 4X solutions in double distilled water: aldicarb, ammonium chloride, copper sulfate, mercuric chloride, methamidophos, nicotine, paraquat dichloride, phenol, sodium arsenite, sodium azide, sodium cyanide and toluene. In addition, sodium azide was tested at 0.1 and 1.0 mg/L, concentrations significant to fish health (5). Sodium hypochloride was tested as a residual chlorine control, at 0.2 and 4.0 mg/L, levels typically associated with municipal drinking water (101). All solutions were made immediately prior to analysis and were diluted to their final concentration upon addition to a well of melanophores. The melanophore responsiveness to each water contaminant was conducted in triplicate.

**Table 2**: Water contaminants tested on *O. tshawytscha* melanophores. Each agent was tested at the military exposure guideline (MEG) and the human lethal concentration (HLC). All chemicals were purchased through Sigma-Aldrich with the exception of phenol, which was purchased through USB.

| Agent | Catalog Number | MEG (mg/L) | HLC (mg/L) |
|---|---|---|---|
| Aldicarb | S-190 | 0.005 | 0.047 |
| Ammonia | A-0171 | 30.0 | 72.6 |
| Copper Sulfate | AC422870050 | 0.14 | 92.9 |
| Mercuric Chloride | RDCM0300-100B1 | 0.001 | 24.1 |
| Methamidophos | 06-721-932 | 0.002 | 1.4 |
| Nicotine | 06-722-003 | 0.13 | 1.87 |
| Paraquat Dichloride | USPST740AS | 0.05 | 3.0 |
| Phenol | 75829 | 3.0 | 65.3 |
| Sodium Arsenite | RDCS0280-100B1 | 0.02 | 1.9 |
| Sodium Azide | S-2002 | 0.1* | 1.0* |
| Sodium Cyanide | AC424300050 | 2.0 | 2.5 |
| Sodium Hypochlorite | AC419550250 | 0.2** | 4.0** |
| Toluene | AC42550250 | 1.0 | 2800.0 |

*Sodium azide was tested at 0.1 and 1.0 mg/L, relevant concentrations for fish health and does not correlate to the MEG and HLC.
**Sodium hypochlorite was tested at 0.2 and 4.0 mg/L, relevant concentrations for residual chlorine levels in drinking water and does not correlate to the MEG and HLC.

Bacterial Strains and Culturing Conditions.

All bacterial strains and plasmids used in this study are listed in Table 3. Plasmid extractions from *Salmonella* and *Escherichia* strains were conducted using QIAprep Spin Miniprep Kit (Qiagen, Cat. No. 27106). *Salmonella typhimurium* genomic DNA was isolated using the Promega Wizard® Genomic DNA Purification Kit (Cat. No. A1120). DNA concentrations were determined using the NanoDrop ND-1000 UV-Vis spectrophotometer (ThermoFisher Scientific, Wilmington, DE, USA).

For melanophore response assays, salmonid bacterial pathogens *Aeromonas salmonicida* SD2, *Yersinia ruckeri* LP4 and *Flavobacterium psychrophilum* LP5 were cultivated in TYES medium [10 g tryptone (Difco), 1 g yeast extract (Difco), 8 g sodium chloride (Difco), 0.3 g calcium chloride (Difco)] at 26°C for 48 hours without aeration, except for *F. psychrophilum* which was grown at 16°C. Additionally, salmonid bacterial pathogen *Carnobacterium piscicola* SD2 was grown in Tryptic Soy Broth (TSB, Difco) at 26°C for 48 hours without aeration.

*Salmonella* and *Escherichia* strains were maintained in Luria-Bertani (LB) broth [10 g tryptone (Difco), 5 g sodium chloride (Difco), 5 g yeast extract (Difco)] supplemented with 20% glycerol. Bacterial stocks were stored at -80°C and fresh cultures were streaked onto LB agar and incubated at 37°C overnight with the exception of IB314 which requires growth at 30°C. Isolated colonies were selected for experimentation and were grown in LB broth at 37°C (30°C for IB314) with aeration overnight. For *Salmonella* erythrophore assays, overnight cultures were subcultured 1:100 in fresh LB media. *Salmonella* supernatants were collected following 0.2 µm syringe filtration. When appropriate, LB was supplemented with the following antibiotics (Sigma-Aldrich): ampicillin (Amp), kanamycin (Kan) and tetracycline (Tet); at 25, 50 or 100 µg/mL. All Biosafety Level 2 (BSL2) bacterial agents were cultured in an approved BSL2 facility and performed under the requirements and regulations of Oregon State University's Institutional Biosafety Committee.

**Table 3**: Bacterial strains and plasmids used in this study.

| Strain/plasmid | Relevant Characteristics | Source |
|---|---|---|
| *A. salmonicida* SD3 | Outbreak isolate | ODFW<br>Oregon State University |
| *Y. ruckeri* LP4 | Outbreak isolate | ODFW<br>Oregon State University |
| *F. psychrophilum* LP5 | Outbreak isolate | ODFW<br>Oregon State University |
| *C. piscicola* SD2 | Outbreak isolate | ODFW<br>Oregon State University |
| *S. typhimurium* ATCC 700720 | LT2 wildtype | ATCC |
| ST8 | Derivative of ATCC 700720, *prgHIJK*::EZ-Tn5 Kan$^r$ | This study |
| ST8 pTA108*prgHIJK* | ST8 with pTA108*prgHIJK* Kan$^r$, Amp$^r$ | This study |
| *S. typhimurium* IB1 | ATCC 14028s wildtype | Fang, F.C.<br>University of Washington |
| IB2 | Derivative of IB1, *rpo*E::Cm | Fang, F.C.<br>University of Washington |
| IB43 | Derivative of IB1, *rpo*S::Tn10dCm | Fang, F.C.<br>University of Washington |
| IB314 | Derivative of IB1, Δ*rpo*H::AP | Fang, F.C.<br>University of Washington |
| *E. coli* JM110 | F' *tra*D36 *lac*IqΔ(*lac*Z)M15 *pro*A+B+/*rps*L (Str$^r$) *thr leu thi lac*Y *gal*K *gal*T *ara fhu*A *dam dcm gln*V44 Δ(*lac-pro*AB) | Schuster, M.<br>Oregon State University |
| *E. coli* GM2929 | F- *ara*-14 *leu*B6 *thi*-1 *fhu*A3 *lac*Y1 tsx-78 *gal*K2 *gal*T22 *gln*V44 *his*G4 *rps*L136 (Str$^r$) *xyl*-5 *mtl*-1 *dam*13::Tn9 (Cam$^r$) *dcm*-6 *mcr*B1 *hsd*R2 (rK-mK+) *mcr*A *rec*F143 | *E. coli* Genetic Stock Center<br>Yale University |
| *E. coli* Top10 chemically competent cells | F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*) 7697 *gal*U *gal*K *rps*L (Str$^r$) *end*A1 *nup*Gλ- | Invitrogen |
| pTA108 | *lac*ZΔM15 *bla* (Amp$^r$) | Lab stock |
| pTnMod-RKm' | R6K RP4 Kan$^r$ | Miller, VL<br>Washington University School of Medicine |
| EZ-Tn5$^{TM}$ | Transposome conferring Kan$^r$ | Epicentre Biotechnologies |
| pBR322 | *rep rop bla* (Amp$^r$) *tet* (Tet$^r$) | NEB |

Melanophore Tissue Analysis

Melanophores were tested for their ability to distinguish between healthy and diseased tissues harvested from rainbow trout. ODFW generously provided healthy rainbow trout as well as rainbow trout infected with *F. psychrophilum*, the causative agent of Bacterial Cold Water Disease (BCWD). Spleens, livers and kidney were dissected from healthy and diseased trout, submerged in 200 µL L15 (without antibiotic) and stored at 4°C until analysis. To prepare tissues for analysis, 5 spleens, 5 livers and 5 kidneys of healthy and diseased trout were pooled and homogenized (separately) with a rolling pin. Liquefied tissue was pipetted into an eppendorf tube and centrifuged for 60 seconds at 14,500 rpm using a benchtop microcentrifuge to remove cell debris. Melanophores were exposed to collected supernatants, and images were captured over 20 minutes as described earlier. This experiment was conducted in triplicate.

Growth Curve and Detection Limit Assay

Overnight *Salmonella* cultures were subcultured 1:100 in fresh LB and incubation was continued at 37°C with aeration in a side armed flask. At one hour intervals, starting with subculture time zero, the $OD_{600}$ was recorded, 75 µl of culture was removed for erythrophore analysis and 100 µL was removed for a viable plate count. Experimental analysis continued for up to 8 hours. In addition, erythrophores were exposed to the overnight culture, and a viable plate count was also determined. Erythrophore aggregative response was thus correlated to the *Salmonella* growth curve.

To delineate between the critical stimulus, cell number or culture age for erythrophore aggregation, late log cultures were serial diluted, added to erythrophores and analyzed as previously described.

Polymerase Chain Reactions

PCR was conducted using either a Perkin Elmer Gene Amp PCR system (model 2400) or Techne Techgene® PCR machine. Samples were set up using 50-100 ng DNA, 0.8 µM each primer (Table 4) and Platinum® PCR Super Mix HiFi (Invitrogen

Cat. No. 11306016) to a final volume of 50 μL. Negative controls lacking DNA template and positive controls with known pertinent products were used when necessary. PCR reactions were subject to 95°C for 5 minutes to ensure complete initial denaturation, and then went through 30 cycles of denaturing (95°C, 30 seconds), annealing (set at 5°C below lowest $T_m$, 30 seconds) and extension (72°C, 1 minute for each kb). A final extension was conducted for 15 minutes to make sure that all products were adequately extended by the polymerase. To verify the presence of an amplified product, PCR samples were analyzed by 1% agarose gel electrophoresis in TAE buffer (0.8 M tris-base, 0.35 M glacial acetic acid, 20 mM EDTA). DNA bands were visualized by ethidium bromide staining. When necessary, PCR products were purified using QIAquick® PCR Purification Kit (Qiagen, Cat. No. 28104) or DNA bands were excised from the gel using Qiagen Gel Extraction Kit (Cat. No. 28704).

**Table 4**: Primers used in this study. All primers were purchased through IDT.

| Primer Name | Sequence (5'- 3') | Target | $T_m$ (°C) | Fragment Size (bp) | Reference |
|---|---|---|---|---|---|
| KAN-2 FP-1 | ACCTACAACAAAGCTCTCATCAACC | EZ-Tn5 | 63 | Varies | Epicentre |
| KAN-2 RP-1 | GCAATGTAACATCAGAGATTTTGAG | EZ-Tn5 | 60 | Varies | Epicentre |
| EZ-Tn5 Kan F | GCTCAGGCGCAATCACGAATGAAT | Kanamycin | 60 | 500 | This study |
| EZ-Tn5 Kan R | TTTATTCAACAAAGCCGCCGTCCC | Kanamycin | 60 | 500 | This study |
| M13F | GTAAAACGACGGCCAG | Varies | 55 | Varies | Invitrogen |
| M13R | CAGGAAACAGCTATGAC | Varies | 55 | Varies | Invitrogen |
| ST8-prgH-CDS-1 | GGATGCGACCGAAATTATACT | *prgHIJK* | 53 | Sequencing | This study |
| ST8-prgH-CDS-1 RevComp | AGTATAATTTCGGTCGCATCC | *prgHIJK*-EZ-Tn5 | 53 | Sequencing | This study |
| ST8-prgH-CDS-2 | TACCGCAGCAGGCCAGGCGGA | *prgHIJK* | 69 | Sequencing | This study |
| ST8-prgH-CDS-3 | GAATGCCGTTATAGGGCAGGC | *prgHIJK* | 59 | Sequencing | This study |
| ST8-prgH-CDS-4 RevComp | GTTAAGTATCCATAACGTCC | *prgHIJK*-EZ-Tn5 | 48 | Sequencing | This study |

**Table 4 (Continued)**: Primers used in this study. All primers were purchased through IDT.

| **Primer Name** | **Sequence (5'- 3')** | **Target** | **$T_m$ (°C)** | **Fragment Size (bp)** | **Reference** |
|---|---|---|---|---|---|
| ST8-promoter-1 | GATTCCCTGATGAAAATAGAATG | *prgHIJK* | 50 | Sequencing | This study |
| ST8-promoter-2 | CAGACCATTGCCAACACACGC | *prgHIJK* | 60 | Sequencing | This study |
| ST8-promoter-2 RevComp | GCGTGTGTTGGCAATGGTCTG | *prgHIJK*-EZ-Tn5 | 60 | Sequencing | This study |
| ST8-promoter-3 | TGAAGAGGTCAATGGCCACATGG | *prgHIJK* | 60 | Sequencing | This study |
| ST8-promoter-3 RevComp | CCATGTGGCCATTGACCTCTTCA | *prgHIJK*-EZ-Tn5 | 60 | Sequencing | This study |
| prgHIJK-EcoRI-F | GCGAATCGTTCCTTACTGGTATCCTACTGA | *prgHIJK* | 61 | 3000 | This study |
| prgHIJK-SalI-R | TCGAGTCGACCATTCCGGCGCTATCTGC | *prgHIJK* | 68 | 3000 | This study |

Bacterial Transformations

Transformations in *E. coli* were performed following a chemical competency protocol. Cultures were brought to mid-log phase and 2 mL of culture was spun down in a microcentrifuge at maximum speed for 1 minute. Supernatant was decanted and cells were resuspended in the residual medium. Then, 200 µL of cold TSS (10 g PEG 3350 or 8000, 0.5 g $MgSO_4 \bullet 7H_2O$, 88 mL LB, 5 mL DMSO) was added to the cell suspension, which was subsequently alliquoted; 100 µL for each transformation. Cells were kept on ice while adding 1-5 µL of plasmid DNA. After gently flicking the tube, cells were incubated on ice for 20 minutes. The cells were heat shocked at 42°C for 90 seconds and promptly returned to ice for 3 additional minutes. Cells were allowed to recover with the addition of 900 µL of LB followed by incubating at 37°C for 1 hour. The transformed culture was then diluted 1:10 and 1:100 with 50-300 µL of each dilution used to plate on the appropriate selective media.

Transformations in *S. typhimurium* were performed following an amended electrocompetency protocol (204). To prepare electrocompetent cells, 10 mL of overnight culture was diluted into 1 L LB (1:100) and transferred to a 2 L flask. The culture was incubated at 37°C with heavy aeration and pulled during late log phase ($OD_{600}$ 0.7-0.8). Every step from this point forward was conducted on ice and all reagents and materials were kept on ice in preparation. The culture was equally divided into centrifuge bottles and spun at 5,000 rpm for 15 minutes at 4°C using a Beckman J2-12 centrifuge. Culture supernatant was discarded and cell pellets were resuspended in 100 mL cold sterile water. The cells were spun again at 5,000 rpm for 15 minutes at 4°C. The supernatant was discarded and cell pellets were resuspended in 50 mL cold water and consolidated into two centrifuge bottles. The cells were washed and centrifuged again under the same conditions, and the cells were resuspended in 25 mL cold transformation buffer (272 mM sucrose, 15% glycerol). Following the final centrifugation at 5,000 rpm for 10 minutes at 4°C, the cell pellets were weighed and resuspended in cold transformation buffer at a volume equal to 1.5 times the weight (i.e. a cell pellet weighing 1 g would be resuspended in 1.5 mL). At this stage, cells were electrocompetent and were either snap frozen in a dry ice/ethanol bath and stored at -80°C or immediately used for electroporation. Fifty µL

of electrocompetent cells were used for each electroporation. On ice, 50-100 ng DNA was added and mixed by pipetting. After a 1 minute incubation on ice, cells were transferred to a 0.2 cm electrocuvette and tapped to the bottom of the cuvette. Using a BioRad Gene Pulser set at 2.5 kV, 25 μF and 400 Ω, a single electric pulse was delivered to the electrocompetent cells. Typically a time constant between 4-8 ms was observed. Immediately after discharge, 1mL of room temperature SOC (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl2, 10 mM MgSO4, 20 mM glucose) was added and cells were allowed to recover at 37°C with aeration for 1-3 hours depending on the plasmid copy number. Cultures were diluted and plated on selective media. Often, plates were incubated for longer than 24 hours before colonies were visible.

Transposon Mutagenesis

*Salmonella typhimurium* ATCC 700720 was mutagenized using the EZ-Tn5™ <KAN-2>Tnp Transposome™ Kit (Epicentre Biotechnologies, Madison, WI, USA, Cat. No. TSM99K2). Electrocompetent cells were prepared as described. On ice, 1 μL EZ-Tn5™ transposome™ and 1 μL TypeOne™ Restriction Inhibitor (Cat. No. TY0261H) was added to 50 μL electrocompetent cells. After mixing gently by flicking the tube, cells were incubated on ice for 1 minute. Electroporation was carried out as previously described and cells were recovered at 37°C with aeration for 1 hour. The EZ-Tn5™ transposase was activated by an increase in $Mg^{2+}$ concentration within the intracellular environment. Successful transposition events were screened by plating on LB Kan 50 μg/ml. Transposants were stored as top agar (10 g tryptone, 5 g yeast extract, 5 g NaCl, 7 g agar, 1 L $H_2O$) stabs and kept at 4°C until further analysis.

Transposants were screened on *B. splendens* erythrophores by first starting overnight cultures in LB Kan 50 μg/ml, subculturing 1:100 and bringing the cultures to late log phase. Transposant cultures were diluted 1:10 upon addition to a well of erythrophores maintained in L15+ media. This process was first crudely conducted by observing erythrophore response under 40X magnification using an Olympus Optical CK2 inverted microscope. Cultures that did not induce pigment aggregation within 60

minutes were saved as putative mutants and were subsequently screened again by capturing time lapsed images and analyzing these images as previously described. Putatives with confirmed mutant phenotypes were molecularly processed in order to locate the site of transposome™ insertion.

Sequencing

Sequencing to find the transposome™ insertion site in each *S. typhimurium* mutant was conducted by the Center for Genome Research and Biocomputing (CGRB, Oregon State University) on an ABI 3730 capillary sequence machine. To identify the transposome insertion site, two methods were employed: (i) genomic DNA was enzymatically digested, self-ligated, used as PCR template and then sequenced, or (ii) direct genome sequencing. The ABI 3730 is capable of sequencing off of genomic DNA, although the failure rate is extremely high.

For self-ligations, genomic DNA extractions were performed as previously described. An array of restriction enzymes that do not cut EZ-Tn5™ were selected for analysis: AflII, BclI, BstXI, KpnI, NheI, PacI, SacI and SpeI. Digests were carried out by the manufacturer's (New England Biolabs) suggested protocol using 500 ng genomic DNA in a 50 µL reaction. Self-ligations were set up as follows: 25 µL digested genomic DNA, 20 µL 10X T4 ligation buffer, 1 µL T4 ligase and 154 µL nuclease free water. This ligation reaction is substantially more dilute than a typical ligation to promote "self-ligation" of the individual DNA fragments thus giving numerous closed circular DNA molecules of varying sizes. Ligations were held at 16°C overnight then heat inactivated at 65°C for 10 minutes. DNA was concentrated using QIAquick® PCR Purification Kit (Qiagen, Cat. No. 28104) or Phase Lock Gels (5Prime, Gaithersburg, MD, USA, Cat. No. 2302820 and 2302830). Concentrated self ligated DNA was used as template in a PCR reaction using primers specific for EZ-Tn5™ reading out of the transposition site and into genomic DNA (Table 4), a positive control PCR reaction amplifying the kanamycin gene was included for each self ligation PCR to verify the presence of EZ-Tn5™. The PCR reactions were set up as follows: 3 µL self ligated DNA, 0.8 µM of each primer and Platinum® PCR SuperMix HiFi (Invitrogen Cat. No. 11306016) to a total volume of 50 µL. Initial

denaturing was allowed for 5 minutes at 95°C followed by 30 cycles of denaturing (30 seconds, 95°C), annealing (30 seconds, 58°C) and extension (4 minutes, 72°C). Final extension was set for 15 minutes. PCR products were run on a 1% agarose gel, and samples with a visible band were purified either by the QIAquick® PCR Purification Kit or excised from the agarose gel and purified using Qiagen Gel Extraction Kit (Qiagen Cat. No. 28704). Purified PCR products (~350 ng) were submitted as template for sequencing using 12 pmol of EZ-Tn5™ specific primers (Table 4) in a total volume of 12 µL, concentrations recommended by CGRB (49). This method was successful in identifying the transposon insertion site for *S. typhimurium* mutant ST8.

For direct genomic sequencing, 2-3 µg genomic DNA plus 50 pmol of sequencing primer (Table 4) in a total volume of 6 µL was submitted to CGRB to be sequenced on the ABI 3730. This method was only successful ~10% of the time.

Complementation

To restore the wildtype phenotype to *S. typhimurium* mutant ST8, the entire *prgHIJK* wildtype operon including its native promoter were cloned and ligated into pTA108. Using primers with engineered restriction sites (Table 4), the ~3 kb operon was amplified. Following restriction digests, *prgHIJK* was ligated to CIP (New England Biolabs, Cat. No. M0290S) treated pTA108 overnight at 16°C. The ligation reaction was then electroporated into ST8 electrocompetent cells, as previously described. As a control, an empty pTA108 was also introduced into ST8. Transformants were selected on LB Amp 25 µg/mL plus 50 µg/mL X-gal and 0.1 M IPTG for blue/white screening. Plasmid preps and appropriate restriction enzyme digests followed by 1% agarose gel electrophoresis was used to confirm the presence of the plasmid (pTA108) and insert (*prgHIJK*).

A complementation assay on *B. splendens* erythrophores was performed to select a ST8-pTA108*prgHIJK* transformant with a complemented phenotype that caused a similar erythrophore response as wildtype *S. typhimurium*.

# Chapter 4

# Results

## Part I: Erythrophore Response to *Salmonella typhimurium*

Erythrophores isolated from *B. splendens* react to *S. typhimurium* by aggregating their intracellular pigment organelles. Overnight cultures of *S. typhimurium* incubated at 37°C with aeration induce aggregation after the onset of 3 to 4 hours. Comparatively, erythrophores exposed to *S. typhimurium* in late log phase aggregate much quicker (Figure 3). Overnight cultures were diluted 1:100, brought to late log phase ($OD_{600}$ ~ 1.5), then applied to erythrophores. Erythrophores responded by aggregating to approximately -30% within 60 minutes. This response was distinguishable from the overnight culture as well as the LB media control, which both caused ~10% pigment dispersion in 60 minutes time.

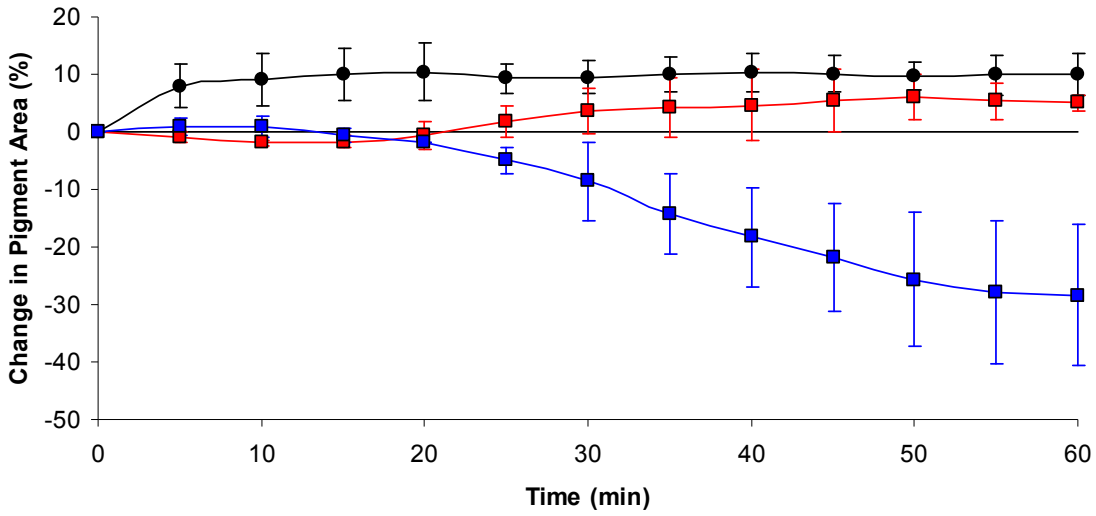


**Figure 3**: Erythrophore response to *S. typhimurium*. Overnight cultures of *S. typhimurium* ATCC 700720 (■) did not cause erythrophore aggregation within 60 minutes. A culture in late log phase (■) induced pigment aggregation. The LB media control (●) induced pigment dispersion. A negative change in pigment area is indicative of aggregation and conversely, a positive change correlates to pigment dispersion. Data represents the average of two trials for each *S. typhimurium* response curve, and an average of three trials for the LB media control. Error bars represent the standard deviation between the experimental trials.

To further investigate the correlation between culture age and erythrophore aggregation rates, a *S. typhimurium* ATCC 700720 growth curve erythrophore analysis was conducted. Overnight cultures were subcultured 1:100 and incubated at 37°C with aeration. Starting with time zero and continuing in 1 hour intervals, the culture $OD_{600}$ was recorded and samples were extracted for viable plate counts and erythrophore analysis (Table 5). Overnight cultures were also tested on erythrophores. This experiment was conducted in duplicate. Erythrophore aggregation initiated at subculture hour 4 with an average change in area of approximately -24% after 60 minutes of exposure. The sample extracted at hour 4 correlated to an average of 8.5 x $10^8$ bacterial cells added to a well of erythrophores. The number of bacterial cells added for subculture hours 3, 4, 5 and 6 were all on the order of $10^8$ cells. Subculture hours 5 and 6 also resulted in erythrophore aggregation with an average change in area of -20% and -15%, respectively. The *S. typhimurium* ATCC 700720 growth curve averaged from two independent trials and selected end point images are depicted in Figure 4. Images A-F show the erythrophore response to *S. typhimurium* after 60 minutes of exposure at each stage of the growth curve. At each hour, a sample was extracted from the continuous culture and applied to a new population of erythrophores. Erythrophores were observed the following day to check for aggregation, particularly for overnight cultures, time zero and subcultures 1, 2 and 3, which exhibited little to no aggregation within 60 minutes of exposure. Overnight cultures had caused complete aggregation (~ -80%) while time zero and subcultures 1 and 2 never exhibited noticeable aggregation. Subculture hour 3, after roughly 20 hours of exposure, caused complete aggregation (~ -80%) in one trial but only partial aggregation (~ -30%) in the other. As expected, subculture hours 4, 5 and 6 showed complete erythrophore aggregation in both trials after nearly 20 hours of exposure.

**Table 5**: Erythrophore response correlated to the *S. typhimurium* growth curve. Samples from a continuous culture of *S. typhimurium* ATCC 700720 were pulled at 1 hour intervals and tested on erythrophores. Optical density was recorded and viable plate counts were conducted. Number of cells added is an estimation based on the CFU/mL of the culture and refers to the number of bacterial cells exposed to erythrophores. Degree of aggregation was assessed over 60 minutes for each sample. A negative percent change in area corresponds to pigment aggregation and a positive percent change in area is indicative of pigment dispersion. Data represents the average of two trials.

| Time (hrs) | $OD_{600}$ | CFU/mL | Number of cells added | Area Change at 60 min |
|---|---|---|---|---|
| 0 | 0.034 | $4.29 \times 10^7$ | $3.2 \times 10^6$ | 9% |
| 1 | 0.066 | $4.65 \times 10^7$ | $3.5 \times 10^6$ | 7% |
| 2 | 0.309 | $1.69 \times 10^8$ | $1.3 \times 10^7$ | 6% |
| 3 | 0.943 | $1.71 \times 10^9$ | $1.3 \times 10^8$ | -1% |
| 4 | 1.510 | $1.13 \times 10^{10}$ | $8.5 \times 10^8$ | -24% |
| 5 | 1.650 | $9.33 \times 10^9$ | $7.0 \times 10^8$ | -21% |
| 6 | 1.680 | $9.79 \times 10^9$ | $7.3 \times 10^8$ | -16% |
| Overnight | - | $1.28 \times 10^{10}$ | $9.6 \times 10^8$ | 5% |

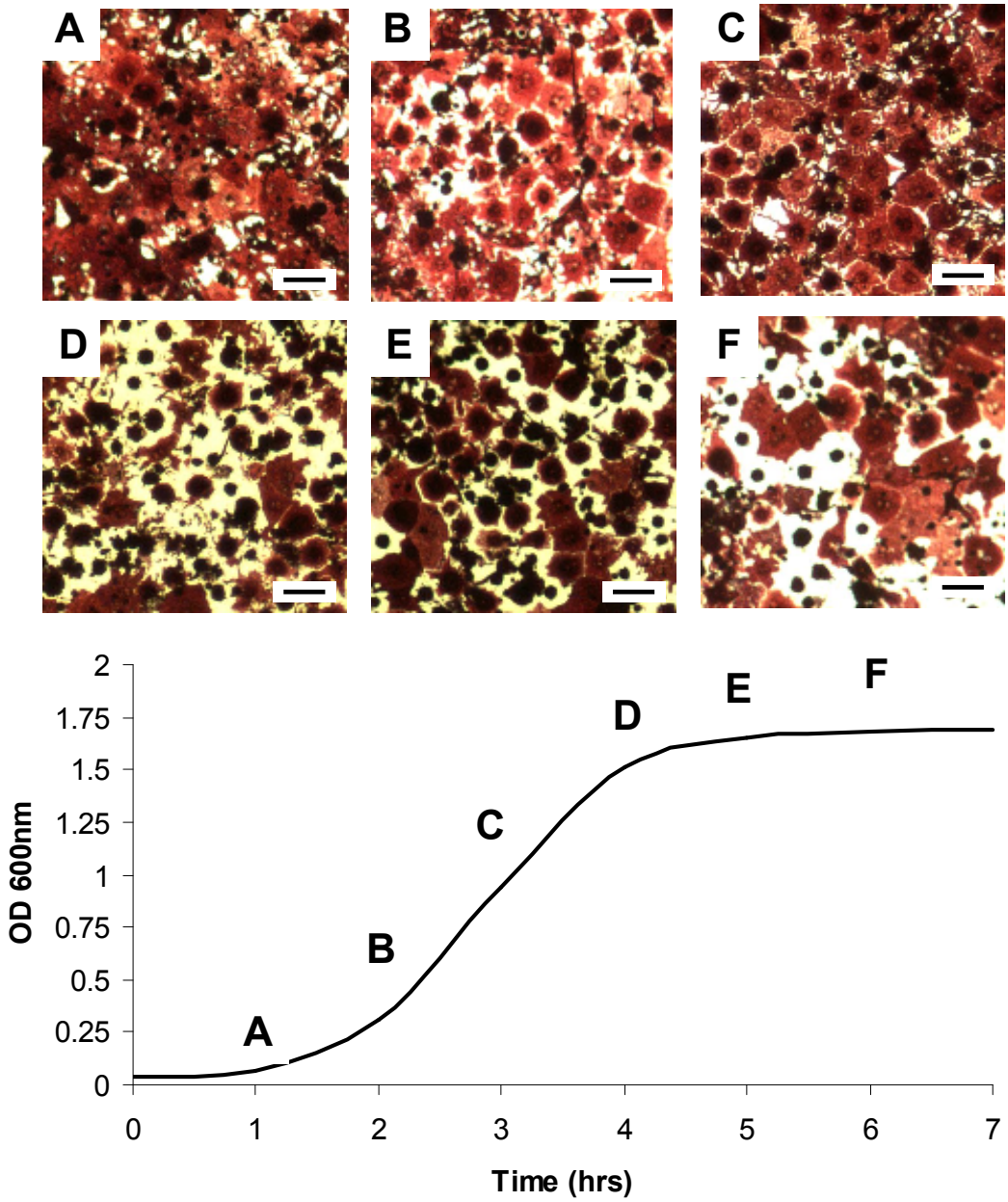


**Figure 4**: Erythrophore response correlated to the *S. typhimurium* growth curve. Samples from a continuous culture of *S. typhimurium* ATCC 700720 were pulled at 1 hour intervals and tested on erythrophores. Degree of aggregation was assessed over 60 minutes for each sample. Images depict erythrophore response after 60 minutes of exposure for samples pulled at subculture (**A**) hour 1, (**B**) hour 2, (**C**) hour 3, (**D**) hour 4, (**E**) hour 5, and (**F**) hour 6. Size bars signify 50 µm. Letters corresponding to each image are represented on the *S. typhimurium* growth curve. Erythrophore aggregation initiated at hour 4 with approximately 7.5 x $10^9$ cells present. Similar aggregative trends were observed for hours 5 and 6. Pigment dispersion was observed at hours 1, 2 and 3. Data represents the average of two trials.

*Detection Sensitivity*

Sensitivity of erythrophores to late log phase *S. typhimurium* was investigated to determine the minimum of bacteria needed to induce pigment aggregation. An overnight culture was diluted 1:100 in LB and incubated further at 37°C with aeration until late log phase ($OD_{600}$ ~ 1.5). Serial dilutions of *S. typhimurium* culture were done in LB medium. Each dilution was immediately added to an individual well of erythrophores (diluted again, 1:10 upon addition to cell culture medium). Images were captured at time zero and at 60 minutes. Only the undiluted culture ($10^0$) and the $10^{-1}$ dilution induced erythrophore aggregation within 60 minutes. After 2 hours of observation, the remaining dilutions still had not caused erythrophore aggregation. Viable plate counts were conducted and it was determined that the $10^{-1}$ dilution resulted in erythrophore exposure to 4.7 x $10^7$ cells. Erythrophores were unable to aggregate in the presence of $10^6$ or fewer cells. This sensitivity experiment was conducted once.

*Erythrophore Response is Cell-associated*

Previous work in the Trempy lab showed that *B. splendens* erythrophore aggregation in response to *Bacillus cereus* ATCC 49064 was dependent on secreted virulence factors found in the culture supernatant (133). Secreted virulence factors are not a prominent feature of *Salmonella* pathogenesis, therefore, it was suspected that the bacterial cell was responsible for inducing erythrophore aggregation. To test this, erythrophores were exposed to late log phase whole culture and 0.2 μm filtered culture supernatant. Additionally, erythrophores were exposed to a bacterial culture which had cells pelleted and subsequently resuspended in fresh LB to determine if erythrophore aggregation was bacterial cell contact dependent. Whole cultures induced aggregation as expected but *S. typhimurium* cells did not cause erythrophore aggregation, nor did filtered supernatant, within 60 minutes of exposure (Figure 5). Following erythrophore image analysis, it was observed that *S. typhimurium* cells did initiate aggregation after approximately 2 hours, although filtered supernatants never initiated aggregation (data not shown). This experiment was conducted once.

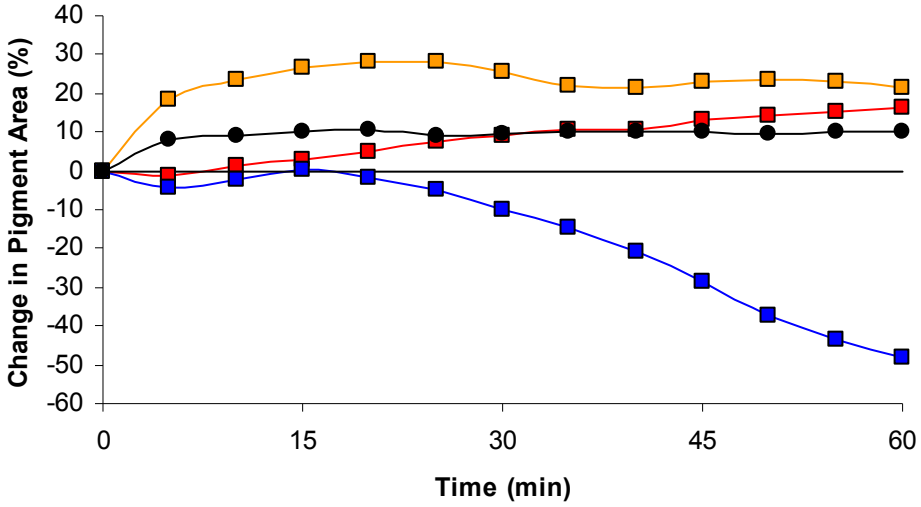


**Figure 5**: Erythrophore response is cell-associated. Late log phase cultures of *S. typhimurium* ATCC 700720 whole culture (■), 0.2 µm filtered culture supernatant (■), and cells (■) resuspended in fresh LB, were exposed to erythrophores. All samples were grown in LB (●) at 37°C. A positive change in area is indicative of pigment dispersion whereas a negative change correlates to pigment aggregation. Data represents a single trial.

*Nutrient Deprivation Assay*

Due to the observation that erythrophores aggregate to *S. typhimurium* ATCC 700720 cultures in late log phase, the potential role of nutrient deprivation was investigated. *Salmonella typhimurium* alternative sigma factor mutant strains IB43 [Δ*rpoS* ($\sigma^S$)], IB2 [Δ*rpoE* ($\sigma^E$)] and IB314 [Δ*rpoH* ($\sigma^H$)] were kindly provided by Ferric C. Fang (University of Washington). Mutant strains were incubated at 37°C with the exception of IB314, which was incubated at 30°C because the heat shock response cannot be activated with a mutant $\sigma^H$. Late log phase cultures in LB were tested on erythrophores as described. The wildtype background strain IB1 (*S. typhimurium* ATCC 14028s) caused erythrophore aggregation similar to *S. typhimurium* ATCC 700720 (Figure 6). Likewise, strain IB2 which has an inactivated $\sigma^E$ produced an erythrophore response curve similar to wildtype, aggregating to -45% within 60 minutes. Strain IB43, the $\sigma^S$ mutant, also caused erythrophore aggregation but was delayed slightly from wildtype in terms of initiation of aggregation. Erythrophores reached -25% after 60 minutes of exposure to IB43. The mutant $\sigma^H$

strain, IB314, caused pigment dispersion similar to the LB control. This experiment was conducted once.

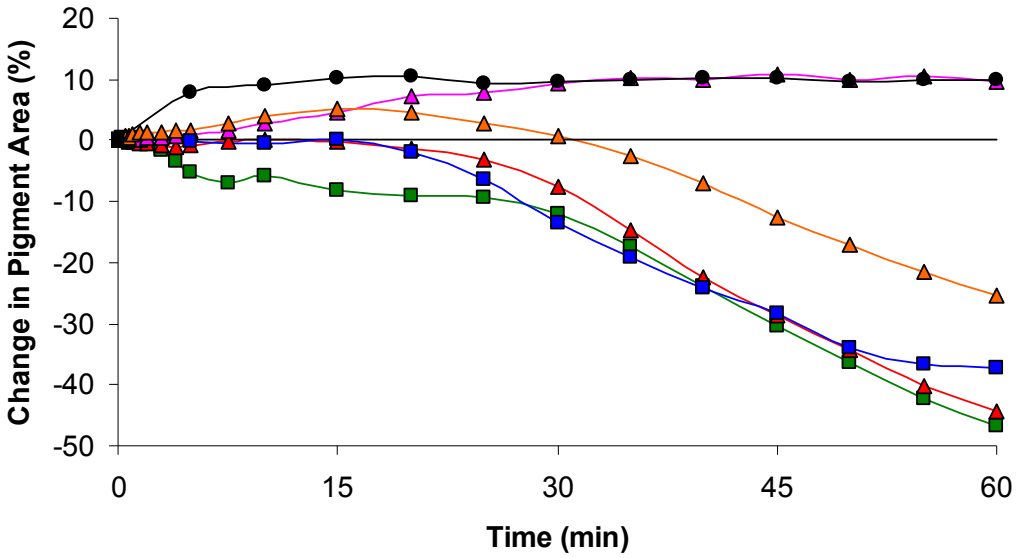


**Figure 6**: Erythrophore response to alternative sigma factor mutants. Late log phase cultures of wildtype *S. typhimurium* IB1 (■), IB2 Δ*rpoE* (▲), IB43 Δ*rpoS* (▲), IB314 Δ*rpoH* (▲) and wildtype ATCC 700720 (■) were added to erythrophores. All strains were cultivated in LB (●) at 37°C with the exception of IB314 (30°C). A positive change in area is indicative of pigment dispersion whereas a negative change correlates to pigment aggregation. Data represents a single trial.

*Identification of Bacterial Factor(s) Responsible for Erythrophore Aggregation*

To identify the bacterial factor(s) responsible for inducing *B. splendens* erythrophore aggregation, *S. typhimurium* ATCC 700720 was transformed with the transposome EZ-Tn5™. An initial screen of 81 *S. typhimurium* EZ-Tn5™ transformants on erythrophores resulted in 16 putative mutants ST1, ST2, etc., that failed to induce pigment aggregation. Image analysis of the putatives confirmed that mutant ST8 failed to induce pigment dispersion through 3.5 hours of exposure but did eventually aggregate sometime thereafter. *Salmonella typhimurium* ATCC 700720 mutant ST8 was chosen for further analysis. Late log phase cultures of ST8 induce erythrophore pigment dispersion of approximately 40% within 60 minutes (Figure 7), showing greater dispersion than the LB media control.

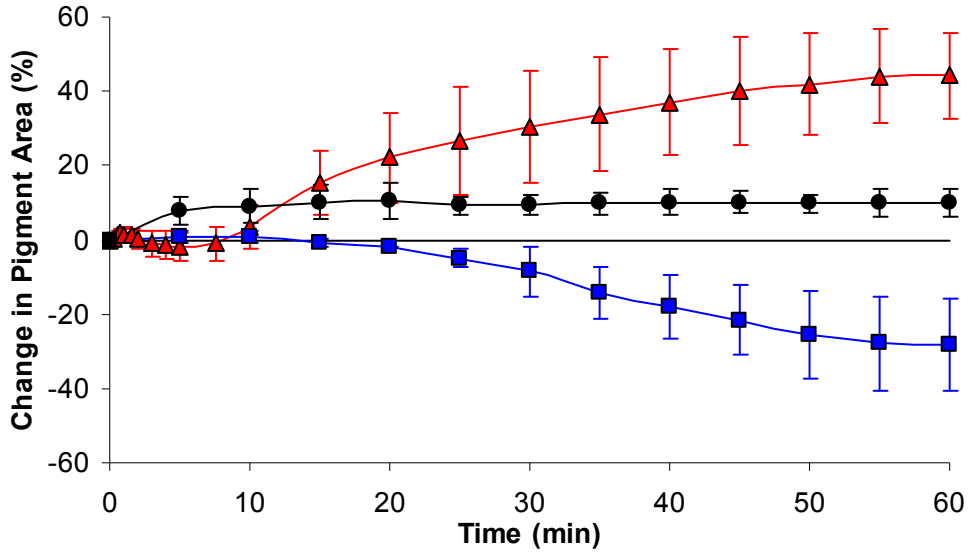


**Figure 7**: Erythrophore response to *S. typhimurium* mutant ST8. Late log phase cultures of *S. typhimurium* ATCC 700720 wildtype (■) and mutant ST8 (▲) cultivated in LB (●) at 37°C were applied to *B. splendens* erythrophores. A positive change in area is indicative of pigment dispersion whereas a negative change correlates to pigment aggregation. Data represents an average of three trials for mutant ST8 and the LB control, the wildtype ATCC 700720 response curve is the average of two trials. Error bars represent the standard deviation between the experimental trials.

*Complementation of the prgHIJK Mutant*

Sequencing analysis of *S. typhimurium* ST8 using EZ-Tn5$^{TM}$ specific primers revealed that the transposome interrupted the promoter region between the -10 consensus sequence and the -35/HilA box sequence of operon *prgHIJK*. To complement the mutant phenotype, the entire *prgHIJK* operon including its native promoter was amplified from wildtype *S. typhimurium* genomic DNA using primers with engineered EcoRI and SalI restriction sites (Table 4). Following EcoRI or SalI digestion, *prgHIJK* was ligated to pBR322 conferring ampicillin and tetracycline resistance and transformed into chemically competent Top10 *E. coli* cells. However, pBR322-*prgHIJK* was never successfully isolated from any transformants, possibly due to the high copy number (~15) of pBR322. Therefore, *prgHIJK* was ligated to low copy number (1-4) plasmid pTA108 conferring ampicillin resistance. The resulting plasmid, pTA108-*prgHIJK* could not be transformed into chemically competent Top10 *E. coli* cells suggesting that *prgHIJK* may be incompatible with or toxic to *E. coli*. *Escherichia coli* do not possess the *prgHIJK* operon and as a result it is suspected that *E. coli* kicked the plasmid out due to its high copy number (in the case of pBR322-*prgHIJK*) and the energy *E. coli* was exhausting on expressing *prgHIJK*. Further, *E. coli* does not express the necessary chaperones for PrgH, PrgI, PrgJ or PrgK to form their macromolecular structure, again resulting in an expendable energy dependent process, perhaps explaining why *E. coli* didn't accept pTA108-*prgHIJK*. Thus, the pTA108-*prgHIJK* ligation was used to directly transform electrocompetent ST8. Transformants were selected on LB Amp 25 μg/mL and successful incorporation of pTA108-*prgHIJK* was determined by plasmid preps and subsequent restriction analysis.

A complementation assay was performed by screening ST8 pTA108-*prgHIJK* transformants on *B. splendens* erythrophores. As a control erythrophores were also exposed to ST8 pTA108. Results showed a successful complementation of the mutant ST8 phenotype which closely resembled the wildtype aggregative response (Figure 8). ST8 pTA108 caused pigment dispersion similar to the LB media control. This assay was conducted once.

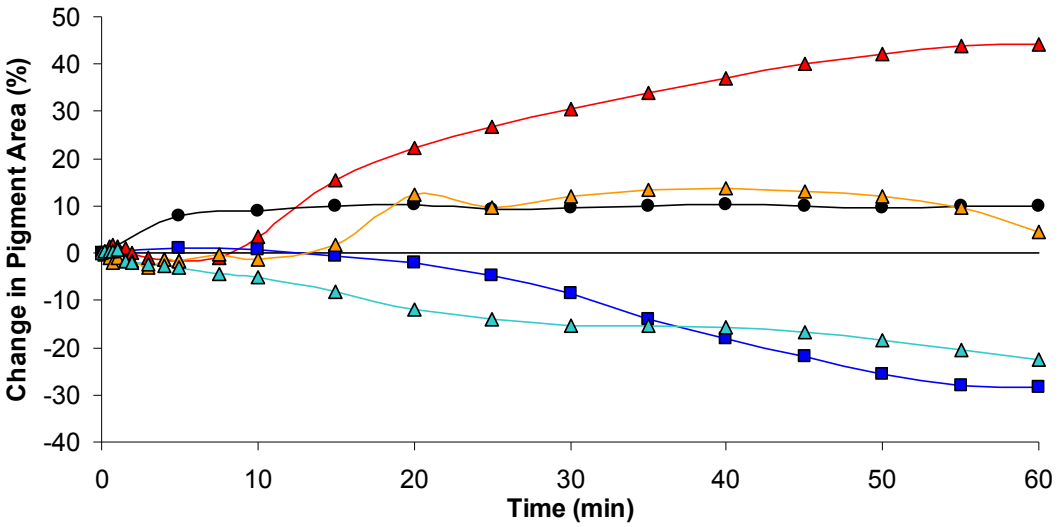


**Figure 8**: Complementation assay. Late log phase cultures of *S. typhimurium* ATCC 700720 wildtype (■), mutant ST8 (▲), ST8 pTA108 (▲) and ST8 pTA108*prgHIJK* (▲) cultivated in LB (●) at 37°C were applied to *B. splendens* erythrophores. A positive change in area is indicative of pigment dispersion whereas a negative change correlates to pigment aggregation. Data represents an average of three trials for mutant ST8 and the LB control, the wildtype ATCC 700720 response curve is the average of two trials, and the response curves for ST8 pTA108 and ST8 pTA108*prgHIJK* represent a single trial.

Part II: Characterization of the *O. tshawytscha* Melanophore

*Melanophore Response to Clonidine*

*Oncorhynchus tshawytscha* melanophores and *B. splendens* erythrophores are similar cell types yet exhibit stark phenotypic differences. Melanophores are brown to black in color, vary in size, increase in proportion to fish length, and can exceed 100 µm in diameter (Figure 9A). Melanophores often show a more dendritic morphology than erythrophores, but can also be present in a sheath-like morphology. Erythrophores are red in color and more consistent in size, averaging around 50 µm in diameter and usually possess a sheath-like morphology, although some dendritic prostheses can be observed (Figure 9B).

In order to characterize the *O. tshawytscha* melanophore in terms of physiological responsiveness, clonidine was used as an aggregative control. *Betta*

*splendens* erythrophores and *O. tshawytscha* melanophores were exposed to 100 nM clonidine, and the response curves of these two cell types were compared (Figure 9C). Pigment aggregation was initiated almost immediately following the addition of clonidine for both melanophores and erythrophores and aggregation was complete for each when pigment could not migrate further. Melanophore aggregation continued for 3 minutes, exhibiting a final percent area change of approximately -75%. Erythrophores responded by aggregating within 2 minutes with roughly a -50% pigment area change. Both melanophores and erythrophores remained in their aggregative state throughout the remainder of the experiment.

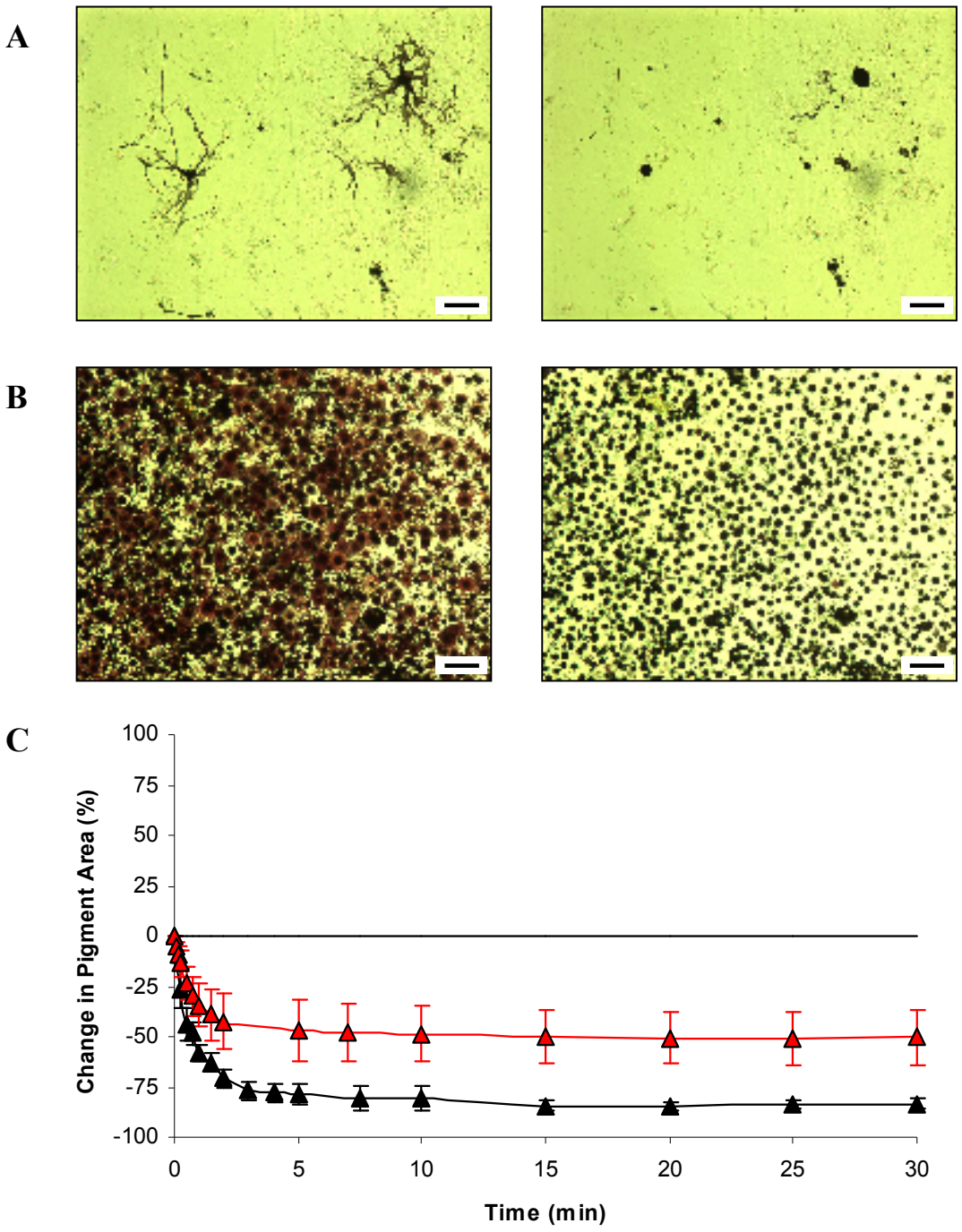


**Figure 9**: Chromatophore response to 100 nM clonidine. **(A)** *Oncorhynchus tshawytscha* melanophores before (left panel) and after (right panel) a 30 minute exposure to 100 nM clonidine at 40X. **(B)** *Betta splendens* erythrophores before (left panel) and after (right panel) a 30 minute exposure to 100 nM clonidine at 40X. Size bar represents 100 µm. **(C)** Graphical representation of pigment aggregation in response to 100 nM clonidine; melanophores (▲) and erythrophores (▲). A negative change in pigment area is indicative of pigment aggregation. Data represents the average of three trials. Error bars represent the standard deviation between the experimental trials.

*Melanophore Response to MSH*

In order to characterize the *O. tshawytscha* melanophore in terms of its pigment dispersion capabilities, α-melanocyte stimulating hormone (MSH) was used as a control and the melanophore and erythrophore reactions to MSH were observed. MSH caused the intracellular pigment organelles to move towards the periphery of the cell, thus melanophores (Figure 10A) and erythrophores (Figure 10B) appear larger. Melanophores were exposed to 100 nM MSH and over a period of 30 minutes, responded by gradually increasing in pigment area to approximately 50% (Figure 10C). Erythrophores followed a similar trend, resulting in roughly a 30% change in pigment area.

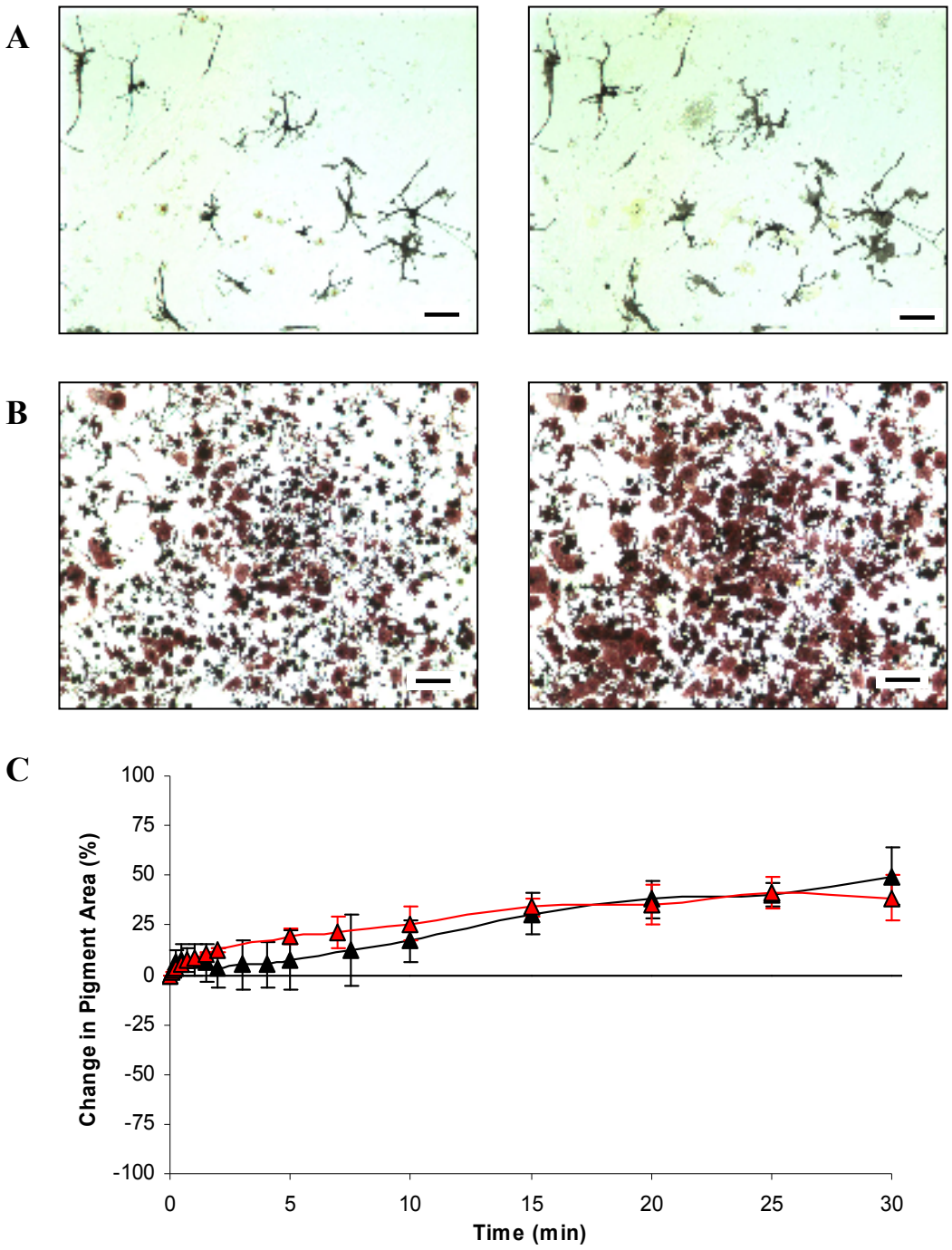


**Figure 10**: Chromatophore response to 100 nM MSH. **(A)** *Oncorhynchus tshawytscha* melanophores before (left panel) and after (right panel) a 30 minute exposure to 100 nM MSH at 40X. **(B)** *Betta splendens* erythrophores before (left panel) and after (right panel) a 30 minute exposure to 100 nM MSH at 40X. Size bar represents 100 µm. **(C)** Graphical representation of pigment dispersion in response to 100 nM MSH, melanophores (▲) and erythrophores (▲). A positive change in pigment area is indicative of pigment dispersion. MSH (α-melanocyte stimulating hormone). Data represents the average of three trials. Error bars represent the standard deviation between the experimental trials.

*Melanophore Longevity in Primary Cell Culture*

To determine how long melanophores remain physiologically responsive in primary cell culture, three melanophore preparations were observed over the course of 6 weeks. Melanophores were exposed to 100 nM clonidine weekly and resulting pigment dynamics were observed (Figure 11). Week 1 data showed pigment aggregation to roughly -85% within 3 minutes, followed by sustained aggregation through 10 minutes. Successive weeks followed a similar trend but aggregated to a slightly lesser degree each week, finishing with week 5 aggregating to approximately -70% within 5 minutes. Data for week 6 could not be obtained because melanophores were observed in an aggregative state (without the addition of clonidine) and were therefore physiologically unresponsive.

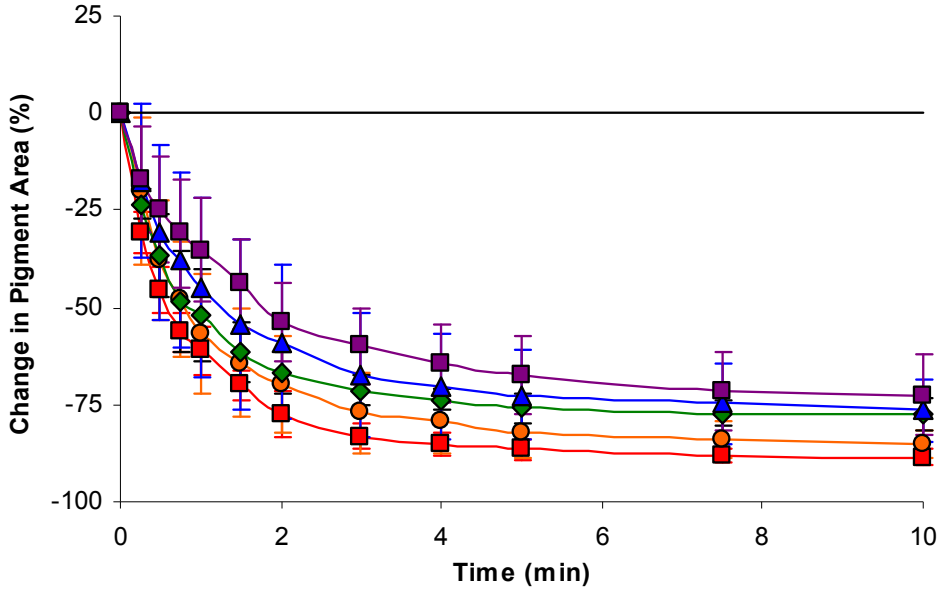


**Figure 11**: Melanophore longevity in primary cell culture. Melanophore preparations were tested for their length of physiological responsiveness in primary cell culture. Three different melanophore preparations were tested weekly. Week 1 (■), week 2 (●), week 3 (◆), week 4 (▲), week 5 (■). By week 6, melanophores were fully aggregated and therefore no longer physiologically responsive. A negative change in area is indicative of pigment aggregation. Data represents the average of three trials. Error bars represent the standard deviation between the experimental trials.

*Melanophore Response to Neurotransmitters*

Melanophores *in vivo* respond to circulating neurotransmitters and hormones by either aggregating or dispersing their pigment organelles which translates to a rapid change in appearance that can be crucial for immediate background adaptation. To further determine melanophore physiological responsiveness in primary cell culture, melanophores were exposed to adenosine, clonidine, dopamine, serotonin and MSH at varying concentrations. Lower limits of detection were determined for each neurotransmitter and the adenyl cyclase activator MSH (Table 6). Adenosine and MSH cause pigment dispersion at 100 nM and 1 nM respectively, while clonidine, dopamine and serotonin cause pigment aggregation correspondingly to 100 nM, 1 μM and 100 μM. Melanophores were observed to aggregate pigment organelles in response to higher concentrations of adenosine.

**Table 6**: Melanophore response to neurotransmitters. Adenosine, clonidine, dopamine and serotonin as well as adenyl cyclase activator α-melanocyte stimulating hormone, MSH were tested at the range indicated in 10 fold increments. All tests were conducted in triplicate.

| Agent | Range Tested | Response | Minimum to Induce Response |
|---|---|---|---|
| Adenosine | 100nM-10mM | Dispersion<br><br>Aggregation | 100nM<br><br>10mM |
| Clonidine | 1nM-10μM | Aggregation | 100nM |
| Dopamine | 100pM-1mM | Aggregation | 1μM |
| Serotonin | 1μM-10mM | Aggregation then recovery | 100μM* |
| MSH | 1pM-1μM | Dispersion | 1nM |

*Aggregation was not induced at higher concentrations.

*Melanophore Response to Water Contaminants*

Melanophores were tested for their ability to respond to toxic water conditions at lethal (human lethal consumption, HLC) and sublethal (military exposure guideline, MEG) doses, as defined by the US Army (2). In total, 12 contaminants were analyzed in addition to a residual chlorine (sodium hypochlorite) control that accounts for typical levels of chlorine in municipal drinking water (Table 7). A control for double distilled water ($ddH_2O$) was also included as all contaminants were diluted in $ddH_2O$. Melanophores were exposed to each contaminate for 1 hour and all experiments were conducted in triplicate. Melanophores were shown to aggregate in the presence of mercuric chloride at the HLC, disperse at the ammonia MEG and aggregate at the sodium arsenite MEG. All other contaminants revealed little to no response from melanophores.

**Table 7**: Melanophore response to common water contaminants. Military exposure guideline (MEG) and the human lethal consumption (HLC) concentrations were tested. NR indicates no response. Dispersion indicates a positive change in pigment area 10% greater than the $ddH_2O$ dispersive response after a 1 hour exposure. Aggregation correlates to a negative change in pigment area less than -10% after a 1 hour exposure. NR indicates no response.

| Agent | MEG (mg/L) | Response | HLC (mg/L) | Response |
|---|---|---|---|---|
| Aldicarb | 0.005 | NR | 0.047 | NR |
| Ammonia | 30.0 | Dispersion | 72.6 | NR |
| Copper Sulfate | 0.14 | NR | 92.9 | NR |
| Mercuric Chloride | 0.001 | NR | 24.1 | Aggregation |
| Methamidophos | 0.002 | NR | 1.4 | NR |
| Nicotine | 0.13 | NR | 1.87 | NR |
| Paraquat | 0.05 | NR | 3.0 | NR |
| Phenol | 3.0 | NR | 65.3 | NR |
| Sodium Arsenite | 0.02 | Aggregation | 1.9 | NR |
| Sodium Azide | 0.1* | NR | 1.0* | NR |
| Sodium Cyanide | 2.0 | NR | 2.5 | NR |
| Sodium Hypochlorite | 0.2** | NR | 4.0** | NR |
| Toluene | 1.0 | NR | 2800.0 | NR |
| $ddH_2O$ | - | Dispersion | - | Dispersion |

*Sodium azide was tested at 0.1 and 1.0 mg/L, relevant concentrations for fish health and does not correlate to the MEG and HLC.
**Sodium hypochlorite was tested at 0.2 and 4.0 mg/L, relevant concentrations for residual chlorine levels in drinking water and does not correlate to the MEG and HLC.

*Melanophore Response to Salmonid Pathogens*

Melanophores were investigated for their ability to respond to salmonid bacterial pathogens *Yersinia ruckeri, Aeromonas salmonicida, Flavobacterium psychrophilum* and *Carnobacterium piscicola* (Figure 12). Melanophore response to all pathogens was distinctive from their respective media control, with the exception of *C. piscicola*. *Yersinia ruckeri* caused the most rapid aggregative response out of the group, reaching -80% within 10 minutes. Similarly, *A. salmonicida* resulted in pigment aggregation to approximately -60% within 15 minutes. The melanophore response to *F. psychrophilum* initiated with pigment dispersion followed by pigment aggregation after 30 minutes of exposure ultimately leading to -75% change in pigment area by 45 minutes time. *Carnobacterium piscicola*, TYES media and TSB media induced minimal changes in pigment redistribution from time zero.

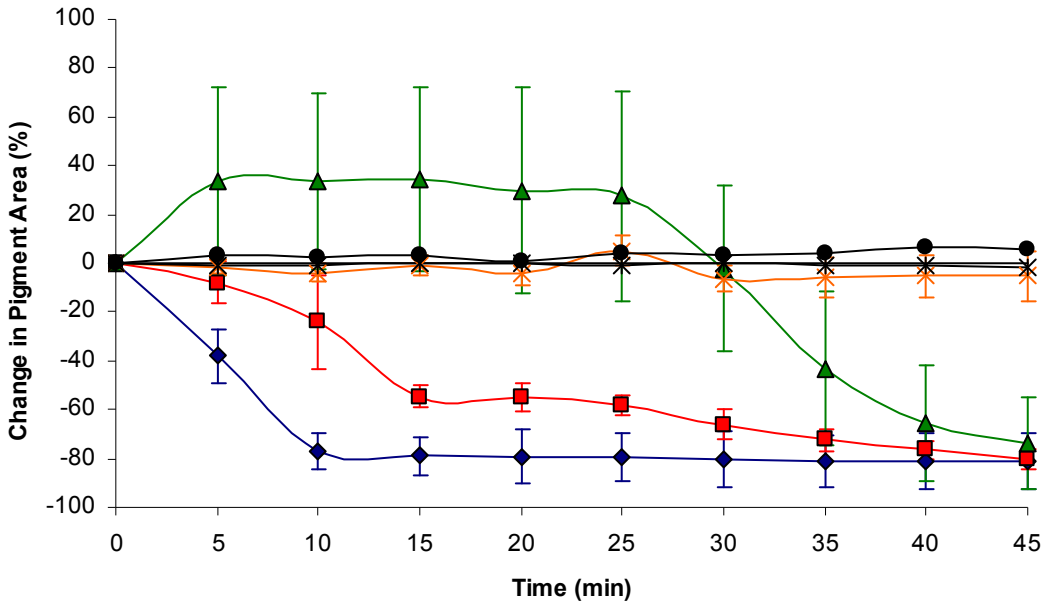


**Figure 12**: Melanophore response to salmonid bacterial pathogens. Cultures of *Flavobacterium psychrophilum* (▲), *Carnobacterium piscicola* (x), *Aeromonas salmonicida* (■) and *Yersinia ruckeri* (◆) were exposed to melanophores. The media controls included TYES (x) and TSB (●). *Carnobacterium piscicola* was grown in TSB and the remaining pathogens were grown in TYES. A negative change in pigment area is indicative of aggregation while a positive change in pigment area indicates pigment dispersion. Data represents the average of three trials. Error bars represent the standard deviation between the experimental trials.

*Melanophore Response to Tissue Samples*

To investigate the potential for melanophores to circumvent a more complex tissue matrix, healthy and diseased organs from rainbow trout were homogenized and added to melanophores. Healthy spleens, livers and kidneys isolated from rainbow trout were compared to spleens, livers and kidneys from rainbow trout infected with *F. psychrophilum* and showing signs of bacterial coldwater disease. Rapid pigment aggregation within melanophores was observed in response to all diseased organs (Figure 13). Diseased spleens resulted in the most rapid pigment aggregation and reached -80% within 4 minutes. Likewise, diseased kidneys approached -80% and diseased livers resulted in a -70% change in pigment area. Healthy kidneys initially induced slight aggregation followed by pigment dispersion to approximately 15% within 20 minutes. Both healthy livers and spleens caused pigment dispersion but did not surpass more than a 10% change.

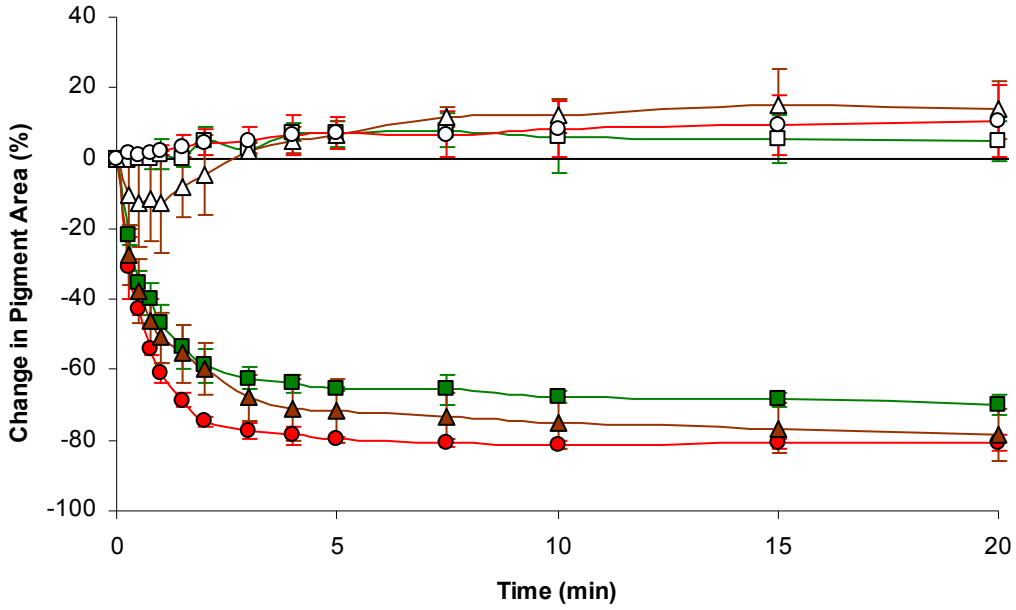


**Figure 13**: Melanophore response to healthy and diseased tissues. Organs from healthy rainbow trout and rainbow trout infected with *Flavobacterium psychrophilum* were collected. Healthy livers (□), healthy spleens (○) and healthy kidneys (△) as well as diseased livers (■), diseased spleens (●) and diseased kidneys(▲) were added to *O. tshawytscha* melanophores. A negative change in pigment area is indicative of aggregation and conversely, a positive change correlates to pigment dispersion. Data represents the average of three trials. Error bars represent the standard deviation between the experimental trials.

# Chapter 5
# Discussion

## Part I: Erythrophore Response to *S. typhimurium*

*Betta splendens* erythrophores are unique cell-based biosensors because they assess the presence of a toxic substance by aggregating or dispersing their pigment organelles, an innate feature of erythrophores. Through optical observations and simple calculations, pigment area is easily quantifiable. This model has the potential to supplement and improve current detection mechanisms because erythrophores report on toxicity rather than the presence or absence of a known structure. *Betta splendens* erythrophores have shown intriguing depth in their ability to respond to different pathogens, such as *Bacillus cereus*, *Clostridium perfringens*, *Clostridium botulinum* and *Salmonella enteritidis*, at varying rates and degrees of pigment movement (133, 134). This suggests that erythrophores respond to different pathogens through diverse mechanisms. *Salmonella typhimurium* has been selected for this study because it accounts for the majority of hospitalizations due to foodborne illness (146, 187, 269).

Erythrophores respond to *S. typhimurium* by aggregating their pigment organelles and this response is distinguishable from the media control, which causes dispersion. Interestingly, an overnight culture causes aggregation within 3 hours, but a culture in late log phase causes this response within 1 hour. This observation prompted a more in depth look at culture age and erythrophore response. Growth curves of *S. typhimurium* in conjunction with erythrophores assays revealed that late log phase cultures proved to cause more rapid erythrophore aggregation than stationary phase cultures. Overnight cultures did cause aggregation but only after a minimum of two hours exposure time. Additionally, mid log phase cultures did not induce erythrophore aggregation, even after an extended exposure time. Altogether, this suggested that the observed erythrophore response was dependent upon culture age. This data led to the hypothesis that the bacterial product(s) responsible for inducing erythrophore aggregation are expressed during late log phase and are not stable for prolonged periods of time.

To further support that erythrophore aggregation was dependent upon culture age, an assessment of erythrophore sensitivity to the number of *S. typhimurium* cells present was conducted. A late log phase culture was serial diluted and each dilution was used in an erythrophore assay. It was determined that a 1:10 dilution of the late log phase culture induced erythrophore aggregation comparable to the undiluted culture. This correlated to erythrophore aggregation with a minimal detection limit of $4.7 \times 10^7$ cells. During the growth curve assessment, mid log phase cultures did not induce erythrophore aggregation and cultures during this phase were associated with $10^7$ to $10^8$ cells on average. This data reaffirms that erythrophore aggregation in response to *S. typhimurium* is indicative upon culture age since approximately the same number of cells are present in two growth phases yet only the older culture causes aggregation. These observations prompted the hypothesis that the bacterial product(s) responsible for inducing erythrophore aggregation may be regulated by a nutrient deprivation response.

Bacterial cultures in late log phase enter stationary phase due to one or more critical nutrients becoming limited and does not permit the continued exponential growth of the bacterium. To remain viable in culture and inhospitable environments, bacteria have adopted different catabolite repression, stress tolerance or alternative growth responses, some of which are critical for *Salmonella* virulence (85, 131, 202, 257). Typically, these responses lead to the expression of a different array of genes that promote varying survival strategies depending on the host or environment that the bacterium occupies. Alternative sigma factors are largely responsible for turning on these genes and contributing to the deactivation of housekeeping gene expression. *Salmonella typhimurium* has 5 known alternative sigma factors with $\sigma^S$, $\sigma^E$ and $\sigma^H$ being the best characterized. The major stationary phase sigma factor, $\sigma^S$, is induced by nutrient limitation and is largely responsible for orchestrating antioxidant defenses (243). Both $\sigma^E$ and $\sigma^H$ enhance the expression of $\sigma^S$ after being induced by extracytoplasmic stress and the heat shock response, respectively (135).

Late log phase mutants in each of these alternative sigma factors were tested on *B. splendens* erythrophores. Since these mutants were created in the background of a different wildtype *S. typhimurium* strain (ATCC 14028s), this was included as a

control along with wildtype *S. typhimurium* ATCC 700720. The wildtype strains induced comparable erythrophore response curves. Mutants in $\sigma^S$ and $\sigma^E$ each caused erythrophore aggregation, with the $\sigma^S$ mutant slightly offset from wildtype aggregation. More alarming was the dispersive response associated with the $\sigma^H$ mutant, which followed the LB control curve nicely. It is important to note that the mutant strain must be incubated at 30°C instead of 37°C. Since $\sigma^H$ is responsible for the heat shock response, the mutant is unable to survive into late log phase at 37°C (249). While $\sigma^H$ may play a role in erythrophore aggregation, the change in incubation temperature could also have played a significant role. Due to the fact that erythrophores respond to $\sigma^S$ and $\sigma^E$ mutants in a manner comparable to wildtype, it was determined that erythrophore response to *S. typhimurium* does not parallel to nutrient deprivation but to some other factor associated with late log phase cultures. To investigate the *S. typhimurium* factor(s) responsible for inducing erythrophore aggregation, random mutagenesis was employed.

*Factors involved in erythrophore aggregation*

Transposome mutagenesis using EZ-Tn5™ was utilized to create a pool of *S. typhimurium* random mutants to identify the bacterial factor(s) involved in erythrophore aggregation. *Salmonella typhimurium* mutant ST8 was identified as an intriguing mutant to study due to its delayed erythrophore aggregation compared to wildtype. Late log phase cultures of ST8 caused erythrophore dispersion that was sustained through at least 4 hours of exposure, a stark difference to late log phase cultures of wildtype *S. typhimurium* which causes erythrophore aggregation within 1 hour of exposure. Therefore, the characterization of mutant ST8 was pursued and it was found that EZ-Tn5™ had interrupted the promoter region of operon *prgHIJK*.

Located within SPI-1, *prgHIJK* forms the major structural components of the *Salmonella* pathogenicity island 1 (SPI-1) type three secretion system (T3SS) apparatus. *Salmonella* are intracellular pathogens and gain access to non-phagocitic host cells through bacterial mediated endocytosis which is orchestrated by the SPI-1 T3SS and by the translocation of bacterial effector proteins through the needle like apparatus and into the host cell cytoplasm. Cytoskeletal rearrangements initiated by

effector proteins promote the uptake of *Salmonella*. Particularly effector proteins SipA and SipC, which bind actin directly and stimulate polymerization (119, 234, 285), while effectors SopE, SopE2 and SopB indirectly promote actin assembly, branching and polymerization by stimulating host Rho GTPases (209, 210). Erythrophore pigment organelles associate with dynein and kinesin motor protein families to travel along the microtubule network. Microtubules composed of tubulin polymers, along with actin microfilaments and intermediate filaments, collectively form the cytoskeleton. If effector proteins are capable of manipulating actin assembly, then they may also be capable of directly or indirectly altering microtubules and intermediate filaments. Interestingly, recent research into organelle transport has revealed a connection between microtubules and actin filaments, and pigment organelles have been observed to switch between different cytoskeletal tracks (221). Myosins, motor proteins associated with actin filaments have been implicated in both pigment aggregation and dispersion, as cells devoid of microtubules are still capable of pigment relocation (235). The evidence presented here suggests that erythrophore aggregation in response to *S. typhimurium* is dependent, at least in part, to the SPI-1 T3SS and is likely to be induced directly through the actions of the SPI-1 T3SS effector proteins.

The data acquired from the cells versus supernatant assay further validates the involvement of the SPI-1 T3SS. Previous studies have shown that *B. splendens* erythrophores aggregate in response to *Bacillus cereus*, and that this response is due to, at least in part, to one or more soluble products present in the bacterial supernatant (133). This same report showed that erythrophores were able to distinguish between the pathogen *B. cereus* and a non-pathogen, *B. subtilis* (133, 134). This lead to the hypothesis that the interaction between erythrophores and *B. cereus* is dependent upon the presence of one or more secreted virulence factors. Gram positive pathogens tend to be associated with secreted toxins more so than Gram negative pathogens, such as *S. typhimurium*. Therefore it was hypothesized that erythrophore aggregation in response to *S. typhimurium* was cell contact dependent and not due to secreted factors found in the culture supernatant. Interestingly, neither *S. typhimurium* cells resuspended in fresh media nor the bacterial supernatant from late log phase cultures

induced erythrophore aggregation within an hour. However, cells resuspended in fresh LB were able to cause aggregation eventually, and did so after approximately 2 hours. Bacterial supernatant caused erythrophore dispersion similar to the LB media control indefinitely. From this data, it can be proposed that the bacterial supernatant of a *S. typhimurium* culture does play a role in erythrophore aggregation. Since bacterial cells exhibit delayed aggregation from whole culture, presumably an important factor is present in the supernatant, yet erythrophore aggregation is largely dependent upon the bacterial cell. Likewise, the bacterial cell alone is not as efficient unless this putative supernatant factor is present. The characterization of *S. typhimurium* mutant ST8 offers an explanation for this quandary. The SPI-1 T3SS injects its needle-like apparatus through the translocon, a pore-like structure that forms on the host cell membrane. The translocon is composed of *S. typhimurium* SPI-1 effector proteins SipB, SipC and SipD. While SipD is associated with the tip of the needle-like apparatus, SipB and SipC exist in the supernatant (151) and do not form the translocon until SipD associates with the host cell membrane (163). All three proteins are critical for translocon formation, and the absence of SipB and SipC would prevent the translocation of the effector proteins responsible for stimulating cytoskeletal rearrangements and therefore, would prevent bacterial mediated endocytosis. Consequently, the delay in erythrophore aggregation observed in response to *S. typhimurium* cells resuspended in fresh LB may be due to the absence of SipB and SipC. Aggregation was eventually observed after 2 hours, giving *S. typhimurium* time to express and secrete SipB and SipC which, along with SipD, form the translocon. Presumably the initiation of erythrophore aggregation occurs following the translocation of the remaining effector proteins. Additionally, needle complex proteins PrgI and PrgJ are also found in culture supernatants (151), and the absence of these proteins may also contribute to delayed erythrophore aggregation.

Mutant ST8 has an insertional inactivation within the promoter region of *prgHIJK*, and therefore, does not express proteins PrgH, PrgI, PrgJ and PrgK. Then in theory, the SPI-1 T3SS apparatus is not assembled because these proteins represent the major structural proteins for this secretion system. Figure 14 shows a depiction of the widltype *S. typhimurium* SPI-1 T3SS as well as a theoretical representation of

mutant ST8. The insertional inactivation of *prgHIJK* should have no effect on the remaining SPI-1 T3SS elements. Considering all the data presented in this report, I propose the following model for *S. typhimurium* induced *B. splendens* erythrophore aggregation (Figure 15).

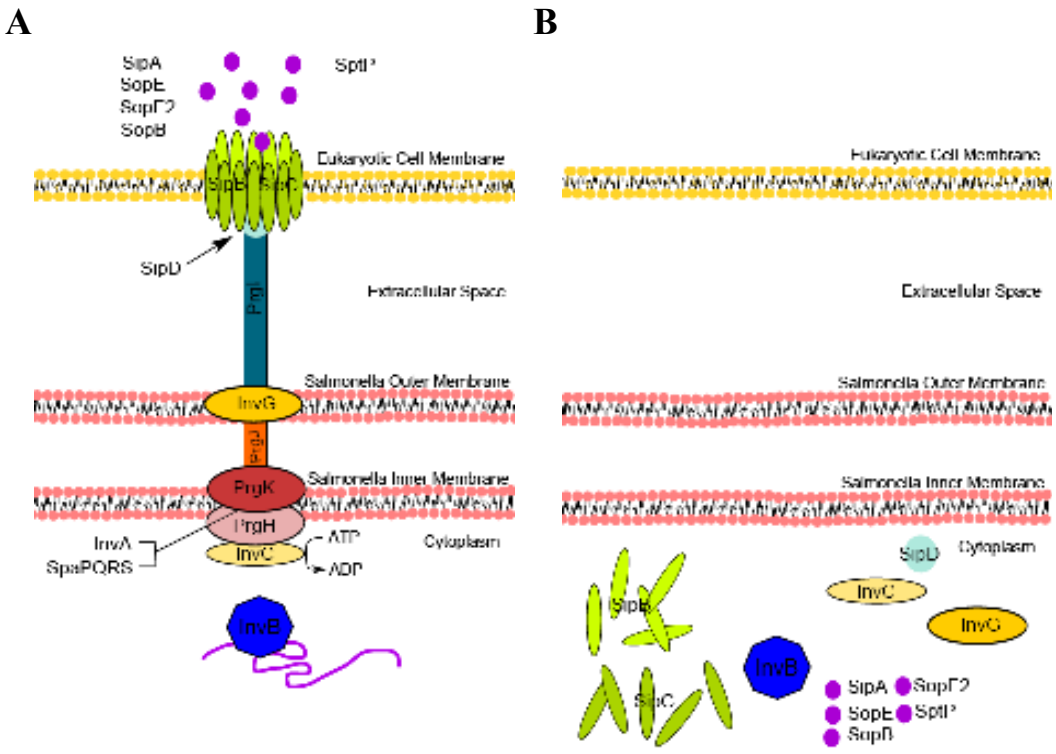


**Figure 14**: Wildtype and mutant ST8 SPI-1 T3SS. (A) Wildtype *S. typhimurium* SPI-1 T3SS apparatus, and (B) mutant ST8 showing a lack of PrgH, PrgI, PrgJ and PrgK expression. The wildtype image shows the delivery of effector proteins (●) via the SPI-1 T3SS, whereas the mutant ST8 cannot translocate effector proteins via the SPI-1 T3SS. See text for details.

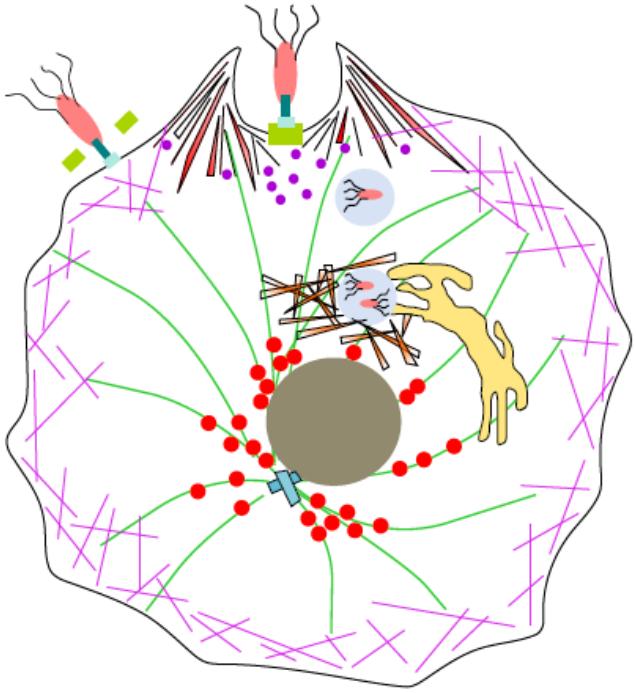

**Figure 15**: Model for *S. typhimurium* induced erythrophore aggregation. Image shows the delivery of effector proteins (●) via the SPI-1 T3SS. Effector proteins induce actin cytoskeletal rearrangement to mediate bacterial-induced endocytosis. Pigment organelles (●) associated with the microtubule network (green) and actin filaments (pink) aggregate to the perinuclear region. Aggregation may result from effector protein activity. Intracellular *Salmonella* replicate within the SCV and form Sifs near the golgi apparatus (yellow). See text for details.

This model merely presents a single pathway towards *S. typhimurium* induced *B. splendens* erythrophore aggregation, albeit a major pathway since mutant ST8 is delayed in inducing aggregation by at least 4 hours. Interestingly, ST8 does induce aggregation sometime after 4 hours. This begs the question, what else could possibly contribute to erythrophore aggregation? One possible explanation is the SPI-2 T3SS compensates for the loss of *prgHIJK* expression. Classically, the SPI-1 and SPI-2 T3SSs have been described as distinctly different and operating during different stages of infection. However, recent research highlights overlapping functions and otherwise deviating activities from the classic segregated view of the two secretion systems (33, 38, 69, 73, 105, 121, 122, 166, 246). For example, the SPI-2 T3SS

shows promise for playing a role in invasion as isolates defective in the SPI-1 T3SS are capable of gaining intracellular access and causing disease in humans and chickens (70, 128). Even if the SPI-2 T3SS doesn't compensate for the loss of the SPI-1 T3SS apparatus in mutant ST8, intriguing activities of an SPI-2 effector protein may contribute to the erythrophore aggregative response to wildtype *S. typhimurium*.

The SPI-2 T3SS effector protein SifA is critical for *Salmonella* induced filament (Sif) formation, but also has a role in blocking kinesin activity (29). Recall that erythrophore pigment organelles are associated with the microtubule network and associate with motor proteins from the dynein and kinesin families to move towards the center and periphery of the cell respecitvely. Microtubules, intermediate filaments and actin filaments together, form the cytoskeletal system. While microtubule polymerization has not been linked to any bacterial effector proteins, SifA, an SPI-2 T3SS effector directly inhibits SCV association with kinesins, which blocks these motor proteins from carrying the SCV to the periphery of the cell (29). SifA interacts with host protein SKIP to allow the SCV to maintain its position in the perinuclear region adjacent to the golgi apparatus (29). SifA may not be responsible for inducing erythrophore aggregation, but SifA may have a role in preventing pigment organelle recovery to the basal state. Perhaps even, SifA is ultimately responsible for complete pigment aggregation and the cytoskeletal rearrangement effector proteins are simply responsible for the initial pigment aggregation, which merely results in ~ -30% area change. SifA could be the catalyst to drive the area change to ~ -60% and less, achieving complete pigment aggregation. These statements are speculative at best, but do open the doors for an expanded investigation into *S. typhimurium* induced *B. splendens* erythrophore aggregation.

*Future Directions for B. splendens Erythrophores*

While it is clear that the SPI-1 T3SS of *S. typhimurium* plays a role in *B. splendens* erythrophore aggregation, there are certainly many unknowns that still remain. First and foremost, alternative pathways leading to erythrophore aggregation are likely to exist given that a *prgHIJK* insertional inactivation did not completely abolish the erythrophore response to wildtype *S. typhimurium*. Second, there are

several SPI-1 effector proteins that have a role in cytoskeletal rearrangements, which could likely be involved in pigment aggregation. Further investigation into each of these proteins will reveal their potential connection to pigment aggregation. Finally, an investigation into the SPI-2 T3SS is warranted given the known association of SPI-2 effector SifA with kinesin motor proteins.

This work provides a foundation for investigating the *B. splendens* erythrophore response to additional Gram negative pathogens that possess T3SSs including *Yersinia* spp., *Shigella* spp., *E. coli*, *Pseudomonas aeruginosa* and *Vibrio* spp., not to mention an analysis on the potential for the involvement of other secretion systems.

*Betta splendens* erythrophores represent an emerging detection method that relies on the innate characteristics of pigment organelle movement. Given a full understanding of the molecular interactions between a bacterial species and the *B. splendens* erythrophore, it would be feasible to engineer an erythrophore cell-based biosensor for specific detection needs. Engineered specificity is not necessarily a desired feature as it may not detect novel or emerging bacterial pathogens. Therefore, *B. splendens* erythrophores may be represented best as innate cell-based biosensors, although in primary cell culture genetic variability also exists between different erythrophore preparations. Likewise, this also has its advantages and disadvantages.

A *B. splendens* erythrophore cell line would undoubtedly take this model detection system to the next level. Erythrophore immortalization would decrease the genetic variability of this cell-based biosensor, which in theory would increase the reproducibility of performing different erythrophore assays. Several techniques to achieve cellular immortalization exist (218), but it will be critical to retain a normal erythrophore phenotype and avoid any changes that may effect pigment organelle movement. Ideally, to counter the loss of genetic variability associated with an erythrophore cell line, it would be advantageous to create several cell lines starting from different erythrophore primary cell preparations. Additionally, it would be prudent to investigate other chromatophore cell types as potential cell-based biosensors because diverse chromatophores may be predisposed for better recognition of particular biological and chemical substances. To some degree, this exploration has already begun by characterizing the *O. tshawytscha* melanophore.

Part II: Characterization of the *O. tshawytscha* Melanophore

For the first time, melanophores isolated from *O. tshawytscha* (Chinook salmon) are described as cell-based biosensors. While this represents a novel model for cell-based biosensors, it also further validates the *B. splendens* erythrophore model proving that it is not a phenomenon, but rather a feature that can be adopted from additional species harboring chromatophores. Why were *O. tshawytscha* melanophores selected as a model to study? Quite simply, the Department of Microbiology at Oregon State University has a long-standing relationship with the Oregon Department of Fish and Wildlife (ODFW), and working with a species important to the Pacific Northwest while continuing this collaboration with ODFW proved to be a wonderful opportunity that could not be ignored. Not to mention, cell-based biosensors have the potential to be implemented as a detection technique for the aquaculture industry, therefore *O. tshawytscha* melanophores seemed like an ideal model to investigate.

Some differences did arise between isolating the chromatophores from their respective species. Melanophores required longer SK washes because there were substantially more epithelial cells to remove, because the fins of 3 to 4 fish were combined into a single prep. Fins of multiple fish were combined because of the low melanophore yield per fin. Even still, substantially fewer melanophores were isolated from the combined fins than from a single erythrophore preparation. This issue along with a perpetual background of some seemingly symbiotic fungal or algal constituents required a slight modification in pigment area analysis. Observation of the background constituents showed minor fluctuations in area occupied with varying samples added to melanophores for analysis. Since these background constituents often associated closely with melanophores, pigment area was calculated for 3 to 4 individual melanophores instead of the entire field of cells. This resulted in a more accurate analysis of the change in pigment area and eliminated the influence of the background constituents on melanophore pigment area. Despite the differences in cell culture preparations and analysis, melanophores responded to aggregative and dispersive controls similarly to erythrophores. However, due to the differences in cell

yield and analysis it is important to describe similarities between melanophores and erythrophores in terms of trends rather than precise area end points.

The data presented here show *O. tshawytscha* melanophores and *B. splendens* erythrophores respond similarly to clonidine, an aggregative control, and MSH, a dispersive control. This indicates that even though these chromatophores are isolated from drastically different fish species, the observed responses are conserved in primary cell culture.

Melanophores were shown to remain physiologically responsive to 100 nM clonidine for up to 5 weeks. Not only are melanophores and erythrophores viable for weeks in primary cell culture but they require very little maintenance. Weekly media changes are sufficient for waste product removal, and chromatophores don't require $CO_2$ to remain metabolically active. These cells are maintained at room temperature, which eliminates any issues concerning assays that would otherwise remove these cells from an incubator. It is intriguing to culture chromatophores from a warm water species (*B. splendens*) and a cold water species (*O. tshawytscha*) and have them tolerate the same primary cell culture conditions.

To further characterize the *O. tshawytscha* melanophore, neurotransmitters at physiologically relevant concentrations were tested. Previous work described the responses of *B. splendens* erythrophores to these same conditions (71). As expected, clonidine, dopamine and serotonin caused pigment aggregation. However, serotonin only induced aggregation at 100 μM, and at this concentration the change in pigment area reached -30% but recovered, nearly back to the basal area within 30 minutes. The inability of melanophores to exhibit a strong aggregative response to serotonin may be due to an altered or lack of serotonergic receptor expression in melanophores. While studies have shown varying serotonergic receptor expression in the fish brain (147, 148), and it has been shown that serotonin does not circulate in the blood of salmon species (181), the presence or absence of such receptors has not been verified in *O. tshawytscha* melanophores. Adenosine and MSH both caused pigment dispersion, but interestingly, melanophores aggregated in the presence of 10 mM adenosine while dispersing at 1 mM, 100 μM, 10 μM, 1 μM and 100 nM which may be the result of an over-saturation event occurring at 10 mM. Overall, the

melanophore response to the selected neurotransmitters was comparable to data previously described for *B. splendens* erythrophores (71), providing further support that pigment response to explicit stimuli is biologically conserved between *O. tshawytscha* and *B. splendens* chromatophores. With this established criteria, *O. tshawytscha* melanophores were investigated for their ability to detect toxic substances.

*Melanophores as Sensors for Water Contaminants*

To address the potential of using *O. tshawytscha* melanophores as sensors for water toxicity, 12 hazardous water contaminants were examined at the MEG (military exposure guideline) and HLC (human lethal level), similar to studies previously conducted with other biosensor systems (15, 264). Additionally, two controls were tested: sodium hypochlorite and $ddH_2O$. Sodium hypochlorite was a control for residual chlorine, and was tested at levels normally found in municipal drinking water. This control did not induce a melanophore response. The $ddH_2O$ control tested the background response, as it was used to dilute each contaminant. Melanophores responded to $ddH_2O$ by dispersing their pigment organelles. Ammonia, at the MEG level of 30.0 mg/L but not the HLC level of 72.6 mg/L also caused melanophore dispersion, but at a pigment area change greater than that experienced with $ddH_2O$. Conversely, mercuric chloride at the HLC level of 24.1 mg/L and sodium arsenite at the MEG level of 0.02 mg/L each caused pigment aggregation. Ammonia, mercuric chloride and sodium arsenite were the only contaminants that provoked a melanophore response distinctive from the controls. A subset of these contaminants were previously tested on *B. splendens* erythrophores, but at elevated concentrations not pertinent to human or fish health (194). The mechanisms underlying melanophore response to ammonia, mercuric chloride and arsenite was not further addressed in this study. The following discussion addresses potential correlations between melanophores and these contaminants while highlighting sources of pollution and known effects on human and fish health.

Ammonia exists naturally in the environment as a product of the nitrogen fixation cycle, and is essential for nucleotide and amino acid synthesis (7). Free living soil

bacterial organisms and symbionts of plant species are the driving forces behind nitrogen fixation, and convert ammonia to nitrates and nitrites which feed back into the cycle. Ammonia is also excreted by many animals and released into the environment from decomposing organic matter. A less significant but natural source of ammonia is the result of volcanic activity. Elevated levels of ammonia in the environment are the result of anthropogenic pollution. Industries involved in fertilizer, steel, petroleum, aquaculture and meat processing contribute to ammonia release but the number one culprit is sewage treatment plants (111, 138). All of these sources have contributed to a homeostatic imbalance in the environment.

Ammonia toxicity affects humans by causing skin or mucosal irritation, depending on the route of entry. An exposure to toxic levels (HLC) of ammonia causes tissue necrosis which occurs as a consequence of an inflammatory response stimulated by phospholipid disruption and cell lysis (4). Low levels of ammonia, such as the MEG, can be tolerated with minimal ill effect. Fish species are susceptible to much lower ammonia concentrations than humans. At sublethal levels on the order of 0.1 mg/L, ammonia acts as an irritant causing skin and gill hyperplasia (240). In primary cell culture, hyperplasia, or the profileration and infiltration of cells, is not possible because primary cell culture implies that the cell are terminally differentiated and incapable of proliferation. Additionally, cells are fixed to a surface and unable to move, therefore, incapable of relocating. Melanophores responded to 30 mg/L ammonia by dispersing their pigment organelles. This reaction, while not hyperplasia by any means, could be viewed as a form of hypertrophy, or the increase in cell size. Fish respond to elevated levels of ammonia greater than 0.1 mg/L by laying on the bottom, acting lethargic and gasping for breath (216). Prolonged exposure, if not treated, ultimately results in death. For unknown reasons, melanophores exposed to 72.6 mg/L ammonia, representing the HLC, did not respond differently from the controls.

Mercury exists in the environment as a natural component in geochemical cycles involving volcanic eruptions, soil erosion and flooding (253). Anthropogenic mercury emissions are the result many industrial practices, with pulp mill and pesticide runoff accounting for the most direct routes to water contamination (79, 239, 253). Mercury

adversely effects human health by interfering with the development and proper function of the immune system (24, 44, 141, 258). Toxic levels or chronic exposure to mercury can lead a plethora of health issues relating to renal damage and neurological dysfunction. One of the most common exposures to mercury is through the consumption of fish (253). Since mercury is largely retained in fish tissues rather than being excreted, mercury bioaccumulates, generally increasing with fish size and matriculating through the aquatic food chain. In fish, mercury enters the bloodstream and binds to hemoglobin (26, 52). The distribution of mercury to different tissues is vast, but mercury has a strong affinity for the kidney (94, 157, 252). Depending on the resident biochemistry, mercury can exist in a number of speciations. Often organic methyl mercury is considered the most toxic, although inorganic mercuric chloride can contribute to tissue damage by traversing lipid bilayers and inhibiting sodium transport (201, 219). In terms of fish health, mercury levels at or below 1 mg/kg body weight are tolerable while levels approaching 10 mg/kg body weight can be lethal (203). Melanophores aggregated in response to 24.1 mg/L mercuric chloride, representing the HLC, but failed to respond to the MEG of 0.001 mg/L. The MEG, or military exposure guideline, represents a concentration that can be tolerated by humans without adverse health effects. The fact that melanophores do not respond to the mercuric chloride MEG but do respond to the HLC indicates that melanophores are ideal sensors to report toxic levels of mercuric chloride while ignoring concentrations unrelated to human health problems. *Oncorhynchus tshawytscha* melanophores show tremendous potential as cell-based biosensors for the detection of mercuric chloride.

Whole fish models are often used to assess the effects of toxic substances, such as mercury exposure. Live *Heteropneustes fossilis* (Stinging Catfish) were observed following mercury exposure by analyzing melanophore morphology (215). Fish were exposed to 0.03 and 0.3 mg/L mercuric chloride and observed over the course of 10 days. The study found that mercury exposure caused melanophore hypertrophy and hyperplasia; an increase in the size of melanophores (i.e. pigment dispersion) and an increase in the number of melanophores in the dermis (215). This evidence corroborated previous studies showing hyperpigmentation in fish exposed to heavy

metals (220, 273). Surprisingly, *O. tshawytscha* melanophores *in vitro* exhibit the opposite effect; pigment aggregation, although this reaction was observed at a much higher mercuric chloride concentration of 24.1 mg/L as compared to the *H. fossilis* study at 0.03-0.3 mg/L. Notably, *O. tshawytscha* melanophores exposed to the MEG of 0.001 mg/L experienced pigment dispersion although this response did not differ from the dd$H_2O$ control.

Arsenic, like ammonia and mercury, is omnipresent in the environment due to natural and anthropogenic sources. In terms of pollution, arsenic is a notable component of herbicides and contaminates waterways as a result of agricultural runoff. In humans, acute arsenic toxicity results in symptoms ranging from gastrointestinal discomfort to death. Arsenic has also been implicated as a causative agent for some cancers, potentially acting by altering DNA methylation and cell proliferation ultimately leading to tumorigenesis (129). Depending on the route of entry, arsenic has been linked to tumor development in the lungs, skin, bladder, liver and kidney (23, 48, 169, 170, 241, 262). Chronic exposure to arsenic causes skin lesions in humans, which can manifest as hyperpigmention or hypopigmentation (48, 280). The effects of arsenic on fish species is not well documented, but fish can accumulate arsenic and therefore serve as a source for human exposure. One report described the use of live fish to address arsenic toxicity employing melanophores as a biomarker (10). Tissues from *Channa punctatus* (Green Snakehead fish) were observed following exposure to 1 mg/L arsenic trioxide. Short term exposures, on the order of days, revealed melanophores fluctuating between pigment dispersion and aggregation and after prolonged exposure (3 months) melanophores closely resembled control fish suggesting that the fish had acclimated to the sublethal dose of arsenic (10). *Oncorhynchus tshawytscha* melanophores aggregated in the presence of 0.02 mg/L (MEG) arsenic but failed to respond to 1.9 mg/L (HLC). Given the observations with *C. punctatus* melanophores, it would be intriguing to monitor *O. tshawytscha* melanophores over a longer exposure time to arsenic. On the other hand, *O. tshawytscha* may have been exposed to low levels of arsenic in hatchery water prior to being selected for this analysis, and therefore desensitized. For that matter,

the contaminants tested on melanophores that resulted in no response could also been due to a prior exposure and subsequent desensitization.

Interestingly, sodium azide did not cause a melanophore response at 0.1 and 1.0 mg/L, which represents the $LC_{50}$ range for fish (5). Phenol and sodium cyanide share the same $LC_{50}$ range for fish as sodium azide, and despite being tested at elevated concentrations to represent critical levels for human health, did not respond differently from the controls. Perhaps, *O. tshawytscha* melanophores would be more sensitive to lower concentrations of these water contaminants, at levels pertinent to fish health rather than human health. Potentially then, *O. tshawytscha* melanophores could be presented as sensors to better suit aquaculture based industries, perhaps as water quality control detectors for hatchery water. To continue the investigation into *O. tshawytscha* melanophores and applications towards the fisheries industry, melanophores were assessed for their ability to respond to bacterial pathogens that cause disease in salmon.

*Melanophores as Sensors for Bacterial Pathogens*

Melanophores were exposed to salmonid bacterial pathogens *Aeromonas salmonicida*, *Yersinia ruckeri*, *Flavobacterium psychrophilum* and *Carnobacterium piscicola*. Each pathogen, with the exception of *C. piscicola*, induced a melanophore response distinctive from the media control. *Aeromonas salmonicida* and *Y. ruckeri* followed similar response curves, inducing rapid melanophore aggregation. Melanophore response to *F. psychrophilum* began with pigment dispersion followed by pigment aggregation after approximately 30 minutes of exposure. The difference in pigment response to each of these pathogens suggests that melanophores react to these bacteria through different mechanisms.

Further differences resulted from melanophores exposed to healthy and diseased organs infected with *F. psychrophilum*. Interestingly, melanophores responded to these diseased organs differently from *F. psychrophilum* culture, yet each of these responses was distinguishable from the healthy organ responses. Spleens, livers and kidneys were considered in this analysis. The fact that *F. psychrophilum* culture and *F. psychrophilum* infected spleens, livers and kidneys resulted in different

melanophore response curves may suggest that melanophores are responding to a *F. psychrophilum* bacterial product that was expressed in the fish host yet not in culture, or alternatively, melanophores may have responded to a fish host product as a result of the fish immune response to *F. psychrophilum*. Regardless of the mechanism of action, melanophores aggregate in the presence of *F. psychrophilum* whether in culture or as an infected tissue sample and the rate of this aggregation is greatly accelerated for the infected tissues. The fact that melanophores are capable of responding to homogenized tissues shows their ability to tolerate a more complex sample, which is appealing in that it potentially eliminates the need to enrich and isolate a sample prior to melanophore analysis.

While the molecular interactions underlying the melanophore response to bacterial salmonid pathogens was not investigated further in this study, some interesting features of these pathogens may help elucidate the potential mechanism(s) of action. Operating on a former hypothesis established with *B. splendens* erythrophores and *Bacillus* spp., that erythrophores are capable of distinguishing between bacterial pathogens and nonpathogenic species (133, 134), an analysis of known virulence factors may elucidate the interactions between melanophores and salmonid bacterial pathogens.

*Aeromonas salmonicida*, a Gram-negative bacterial pathogen, is classically viewed as the causative agent of furunculosis, an often lethal disease that manifests as musculature lesions protruding through the dermis (16). The bacterium has a fairly broad host range and is capable of disseminating and causing systemic disease. Known virulence factors include the S-layer and its associated adhesins (261), siderophores FstC, FstB and HupA (76), superoxide dismutase SodA and SodB (22, 64, 102), glyerophospholipid:cholesterol acetyltransferase (GCAT) and the serine protease AspA (229).

The host range for Gram-negative *Y. ruckeri* is primarily restricted to salmonid species (40), particularly *Oncorhynchus mykiss* (Rainbow trout), and causes enteric redmouth disease (ERM). Just as the name implies, the classic symptom of ERM is a reddening of the mouth and throat caused by subcutaneous hemorrhaging (45). Bacteremia can occur, which allows for dissemination to the kidney and spleen, and

melanosis has also been observed in *Y. ruckeri* infections (98). Geographically, *Y. ruckeri* is largely isolated to the western hemisphere, and has continued to be a problem for Pacific Northwest fish hatcheries (125). Known *Y. ruckeri* virulence factors include biofilm formation (60), extracellular protease Yrp1 (87) and hemolysin YhlB (89) as well as siderophores RucC and RupD (88).

*Flavobacterium psychrophilum* is a Gram-negative bacterial pathogen and the causative agent of bacterial coldwater disease (BCD); characterized by lesions on the fins. It has been observed that fish infected with *F. psychrophilum* appear darker in color, indicating an increase in the number of chromatophores in the dermis and/or pigment dispersion (171, 276, 279). Complete genome sequencing has revealed many putative virulence factors, although only a few have been characterized. Known pathogenic mechanisms include gliding motility encoded by tlpB (12) and biofilm formation (74), although even these virulence factors are poorly understood.

*Carnobacterium piscicola* is a Gram-positive pathogen that can cause varying disease symptoms including internal hemorrhaging, abdominal distension, fluid accumulation, muscle abscesses and septicemia (16). Disease often occurs in post-spawning fish and can be easily misinterpreted as stress responses, although has not been associated with fish mortality. Melanophores were unresponsive to *C. piscicola*.

Interestingly, both *A. salmonicida* and *Y. ruckeri* possess T3SSs that have only been recently described and therefore still remain largely uncharacterized. The T3SS apparatus of *A. salmonicida* is located on a 140 kbp plasmid, while some effector proteins are encoded on the chromosome (41, 42, 250). It has been shown that the T3SS of *A. salmonicida* is critical for virulence (43). The T3SS of *Y. ruckeri* is distinct from the T3SSs of human pathogens *Y. enterocolitica*, *Y. pseudotuberculosis* and *Y. pestis* but still belongs to the same Ysc family of T3SSs (110). Interestingly, the *A. salmonicida* T3SS also belongs to the Ysc family, whose members share homology in the needle-like apparatus (259). In this same study the T3SS of *S. typhimurium* was shown to have a tremendous effect on erythrophore aggregation. Given this data and the presence of similar secretion systems in *A. salmonicida* and *Y. ruckeri*, an intriguing possibility for a similar mechanism to exist between these pathogens and melanophore aggregation presents itself. Surprisingly, *F.*

*psychrophilum* is devoid of T3SSs and T4SSs, which are common features of Gram-negative pathogens. Regardless of the mechanism, the data presented here shows promise for melanophores to be used as cell-based biosensors for the detection of salmonid bacterial pathogens.

Detection of bacterial pathogens in hatchery reared and wild fish is often accomplished through gross pathological examination and histological analysis, although many symptoms are common to a multitude of bacterial diseases. In the absence of routine monitoring and water quality control, often bacterial problems aren't recognized until a mass mortality among the population of fish occurs. Diagnosis has improved with the development of immunological assays and molecular techniques, but these can be costly procedures and samples are often outsourced as this equipment may not be readily available at fish hatchery sites. Phenotypic tests involving the use of selective or differential media are more practical for hatchery use. Here, melanophores show promise as sensors for bacterial pathogens to supplement techniques already in use.

*Future Directions for O. tshawytscha Melanophores*

It is very intriguing to observe the response of *O. tshawytscha* melanophores to water contaminants and bacterial pathogens commonly associated with these fish in hatchery and natural waterways. Melanophores show tremendous promise as cell-based biosensors and may develop into an incredible diagnostic technique for aquaculture based industries. Just as *B. splendens* erythrophores, melanophore based detection will greatly benefit form the development of an immortalized cell line. This work provides the foundational evidence needed to pursue a more in depth analysis of the molecular interactions contributing to melanophore aggregation and dispersion in response to ammonia, mercury, arsenic, *A. salmonicida*, *Y. ruckeri* and *F. psychrophilum*.

# Chapter 6
# Conclusion

This study describes chromatophore cell-based biosensors for the detection of bacterial pathogens and water contaminants. Cell-based biosensors are function-based detectors that use the physiological response of a living cell to sense biologically stimulating agents. The purpose of this study was twofold and the following objectives were formulated: (i) to broaden our understanding of the *B. splendens* erythrophore response to Gram-negative food-associated bacteria by investigating the response to *S. typhimurium*, and (ii) to introduce the *O. tshawytscha* melanophore as a novel cell-based biosensor.

The premise of the first objective spawned from previous data describing the *B. splendens* erythrophore response to *S. enteritidis* (133, 134). Even though this interaction was not investigated on the molecular level, it established precedent for a more in depth analysis, and *S. typhimurium* was chosen in lieu of *S. enteritidis* since it accounts for the majority of hospitalizations due to foodborne illness (146, 187, 269). It was hypothesized that *B. splendens* erythrophores aggregate in the presence of *S. typhimurium*, similar to the response observed with *S. enteritidis*. To investigate this hypothesis, the following questions were asked: (i) do *B. splendens* erythrophores respond to *S. typhimurium*, (ii) if so, can this response be optimized, and (iii) what bacterial products are involved in stimulating the erythrophore response?

Similar to *S. enteritidis*, erythrophores were shown to aggregate in response to *S. typhimurium*. Erythrophore pigment area aggregated to approximately -30% within 60 minutes, and this response was distinguishable, not only from the LB media control, but also from an overnight culture of *S. typhimurium*. Following a growth curve analysis correlating pigment aggregation, erythrophore response to *S. typhimurium* was determined to be growth phase dependent. Late log phase cultures of *S. typhimurium* induced the most rapid erythrophore aggregation, followed by stationary phase cultures, and these responses were distinguishable from the LB media control. Overnight cultures did induce pigment aggregation, although this reaction occurred after the onset of 3 to 4 hours of exposure. As a result of the growth

curve analysis, late log phase cultures were utilized preferentially in the remaining experiments. Erythrophore sensitivity to the minimum number of *S. typhimurium* cells necessary to induce aggregation was determined to be ~$10^7$ cells. To begin the investigation into identifying the bacterial products responsible for inducing erythrophore aggregation, *S. typhimurium* culture supernatants were tested. From this analysis, it was determined that the erythrophore response was bacterial cell dependent. However, *S. typhimurium* cells resuspended in fresh media induced a delayed erythrophore aggregation, suggesting that the culture supernatant contained critical factors to maintain the same erythrophore response as whole culture. To ascertain the bacterial factors necessary to induce erythrophore aggregation, transposome mutatgenesis was employed in order to conduct an unbiased approach. A mutant that caused dispersion which was sustained for at least 4 hours before eventually aggregating was isolated and identified as *S. typhimurium* mutant ST8. Characterization of mutant ST8 revealed that the transposome had interrupted the promoter region of operon *prgHIJK*, which encodes the major proteins of the SPI-1 T3SS apparatus. The delayed aggregation observed with mutant ST8 indicates that the SPI-1 T3SS is a major factor involved in erythrophore aggregation. While the particular components of the T3SS responsible for inducing erythrophore aggregation will be part of a future study, evidence in the literature suggests that effector proteins involved in cytoskeletal rearrangements (SipA, SipC, SopE, SopE2 and SopB) and blocking motor proteins such as kinesins (SifA) are prime candidates for bacterial factors directly involved in erythrophore aggregation. To complement the wildtype phenotype, ST8 was transformed with pTA108-*prgHIJK* which included the native *prgHIJK* promoter. Erythrophores responded to the complemented ST8 mutant similar to wildtype *S. typhimurium*, supporting the involvement of SPI-1 T3SS as a major factor contributing to erythrophore aggregation, albeit, not the only factor involved considering that ST8 causes delayed erythrophore aggregation after the onset of 4 hours. In conclusion, this study shows that the *S. typhimurium* SPI-1 T3SS plays a major role in erythrophore aggregation and warrants a continued investigation into the T3SS effector proteins directly responsible for stimulating erythrophore aggregation.

The second major objective of this study was to investigate *O. tshawytscha* melanophores as a cell-based biosensor. It was hypothesized that the pigment response was biologically conserved between chromatophores of *O. tshawytscha* and *B. splendens* in primary cell culture. To test this hypothesis and provide evidence for the development of an *O. tshawytscha* melanophore cell-based biosensor, the following questions were asked: (i) is the response conserved, and (ii) if so, can the *O. tshawytscha* melanophore be used to detect water contaminants and salmonid pathogens pertinent to the fishery industry?

To address the first question, melanophores were exposed to aggregative and dispersive controls previously used to characterize *B. splendens* erythrophore pigment movement (71). Melanophores responded as expected by aggregating in the presence of clonidine, dopamine and serotonin while dispersing in the presence of MSH and adenosine. Interestingly, melanophore pigment recovered to a basal level after first aggregating to 100 μM serotonin, suggesting that *O. tshawytscha* melanophores may have insufficient serotongenic receptors. Considering that salmon do not circulate serotonin in the bloodstream (181) and have variable serotongenic receptor expression in the brain (148), this result was not surprising. Despite small differences in cell culture preparations and pigment area analysis, melanophore response to the aggregative and dispersive controls provided substantial evidence that the pigment response to these agents is in fact conserved between *O. tshawytscha* melanophores and *B. splendens* erythrophores. To that end, melanophores were further investigated for their potential to be used as cell-based biosensors.

This study characterized melanophores for the detection of chemicals and bacterial pathogens that *O. tshawytscha* may encounter in their environment, whether in the wild or while being raised in fish hatcheries. First, a selection of water contaminants were tested and it was found that melanophores disperse when exposed to sublethal levels (MEG) of ammonia and aggregate to lethal levels (HLC) of mercuric chloride and sublethal (MEG) levels of sodium arsenite. These responses were distinguishable from the residual chlorine and $ddH_2O$ controls and suggest that melanophores could be used as a detector for these water contaminants. Additionally, melanophores were tested for their ability to detect salmonid bacterial pathogens *A.*

*salmonicida*, *Y. ruckeri*, *F. psychrophilum* and *C. piscicola*. Melanophores aggregated in the presence of *A. salmonicida*, *Y. ruckeri* and *F. psychrophilum* but failed to respond to *C. piscicola*. Each aggregative response was distinguishable from the TYES media control. Interestingly, melanophores responded to *F. psychrophilum* by first dispersing before finally aggregating after 30 minutes of exposure. Finally, melanophores can distinguish between healthy and diseased organs harvested from rainbow trout infected with *F. psychrophilum*. Surprisingly, the melanophore pigment response to *F. psychrophilum* infected spleens, livers and kidneys caused rapid aggregation, which was distinct from the *F. psychrophilum* culture response. This data suggested that melanophores were either responding to a bacterial product that was only expressed in the host and not in culture, or melanophores were responding to a host factor that had been produced in response to the invading pathogen, i.e. an immune complex. Regardless of the mechanism, it is intriguing that melanophores are capable of distinguishing between healthy and diseased tissues. The ability of melanophores to circumvent a complex tissue matrix and still respond in a physiological manner that can be distinguished from the appropriate controls suggests that tissues from diseased fish could be assayed with minimal preparations and possibly eliminating enrichment and isolation steps. Altogether, these results warrant the continued investigation into *O. tshawytscha* melanophores to be used as cell-based biosensors. This body of work merely creates a foundation of knowledge and will require further investigation in order to understand the mechanisms behind melanophore response to chemical and biological substances.

Cell-based biosensors such as *B. splendens* erythrophores and *O. tshawytscha* melanophores report on the toxicity of a substance because they rely on a function-based physiological response rather than reporting on the presence of a structure such as a DNA molecule or protein. Prior to this body of work, the interaction between *B. splendens* erythrophores and Gram-negative bacteria was largely uncharacterized. This represents the initial studies into understanding the molecular mechanisms behind erythrophore aggregation in the presence of *S. typhimurium*. This study also described *O. tshawytscha* melanophores as cell-based biosensors for water contaminants and salmonid bacterial pathogens. Future studies will continue to

investigate the interactions between these chromatophores and the chemically and biologically toxic substances they respond to.

# Bibliography


1. 2008. Business Report. National Oceanic and Atmospheric Administration, National Marine Fisheries Service.

2. 2004. Chemical Exposure Guidelines for Deployed Military Personnel. U.S. Army Center for Health Promotion Prevention Medicine (USACHPPM), Aberdeen Proving Ground, MD.

3. Hatchery Wastewater and Effluent Analysis. Pennsylvania Fish & Boat Commission.

4. 2004. Medical Management Guidelines (MMGs) for Ammonia. U.S. Department of Heath and Human Services, Atlanta.

5. Registry of Toxic Effects of Chemical Substances. The National Institute for Occupational Safety and Health

6. 2007. Seafood Consumption Increases in 2006. National Oceanic and Atmospheric Administration.

7. 2004. ToxFAQs for Ammonia. U.S. Department of Health and Human Services, Atlanta.

8. **Aberle, H., Bauer, A., Stappert, J., Kispert, A., Kemler, R.** 1997. Beta-Catenin is a Target for the Ubiquitin-proteasome Pathway. EMBO **16:**3797-3804.

9. **Allen-Austin, D., Austin, B., Colwell, R.R.** 1984. Survival of *Aeromonas salmonicida* in river water. FEMS Microbiol Lett **21:**143-146.

10. **Allen, T., A. Awasthi, and S. V. S. Rana.** 2004. Fish Chromatophores as Biomarkers of Arsenic Exposure. Environmental Biology of Fishes **71:**7-11.

11. **Altier, C., Suyemoto, M., Lawhon, S.D.** 2000. Regulation of Salmonella enterica serovar Typhimurium Invasion Genes by csrA. Infect Immun **68:**6790-6797.

12. **Alvarez, B., P. Secades, M. Prieto, M. J. McBride, and J. A. Guijarro.** 2006. A Mutation in Flavobacterium psychrophilum tlpB Inhibits Gliding Motility and Induces Biofilm Formation. Appl. Environ. Microbiol. **72:**4044-4053.

13. **Aspengren, S., Hedberg, D., Nilsson Skold, H., Wallin, M.** 2009. New Insights into Melanosome Transport in Vertebrate Pigment Cells, p. 245-302.

*In* K. W. Jeon (ed.), International Review of Cell and Molecular Biology, vol. 272. Academic Press.

14. **Aspengren, S., Nilsson Skold, H., Martensson, L.G.E., Quiroga, G., Wallin, M.** 2003. Noradrenaline- and melatonin-mediated regulation of pigment aggregation in fish melanophores. Pigment Cell Research **16:**59-64.

15. **Aurel Iuga, Ethan Lerner, Tommy R. Shedd, and William H. van der Schalie.** 2009. Rapid responses of a melanophore cell line to chemical contaminants in water. Journal of Applied Toxicology **29:**346-349.

16. **Austin B., A., D.A.** 1999. Bacterial Fish Pathogens: Diseases of Farmed and Wild Fish, 3 ed. Springer-Praxis, New York.

17. **Bakowski, M. A., Circulis, J.T., Brown, N.F., Finlay, B.B., Brumell, J.H.** 2007. SopD Acts Cooperatively with SopB During Salmonella enterica serovar Typhimurium Invasion. Cell Microbiol **9:**2839-2855.

18. **Bakshi, C. S. e. a.** 2000. Identification of SopE2, a Salmonella Secreted Protein Which is Highly Homologous to SopE and Involved in Bacterial Invastion of Epithelial Cells. J. Bacteriol. **182:**2341-2344.

19. **Banerjee, P., and A. K. Bhunia.** 2009. Mammalian cell-based biosensors for pathogens and toxins. Trends in Biotechnology **27:**179-188.

20. **Banerjee, P., D. Lenz, J. P. Robinson, J. L. Rickus, and A. K. Bhunia.** 2007. A novel and simple cell-based detection system with a collagen-encapsulated B-lymphocyte cell line as a biosensor for rapid detection of pathogens and toxins. Lab Invest **88:**196-206.

21. **Bang, I. S., Kim, B.H., Foster, J.W., Park, Y.K.** 2000. OmpR Regulates the Stationary-phase Acid Tolerance Response of Salmonella enterica serovar Typhimurium. J Bacteriol **182:**2245-2252.

22. **Barnes, A. C., M. T. Horne, and A. E. Ellis.** 1996. Effect of iron on expression of superoxide dismutase by Aeromonas salmonicida and associated resistance to superoxide anion. FEMS Microbiology Letters **142:**19-26.

23. **Bates, M. N., Smith, A.H., Cantor, K.P.** 1995. Case-control study of bladder cancer and arsenic in drinking water. Am J Epidemiol **141:**523-530.

24. **Baumann, P. C., Smith, I.R., Metcalfe, C.D.** 1996. Linkages between chemical contaminants and tumors in benthic Great Lakes fish. J. Great Lakes Res. **22:**131-152.

25. **Baxter, M. A., Fahlen, T.F., Wilson, R.L., Jones, B.D.** 2003. HilE Interacts with HilD and Negatively Regualtes hilA Transcription and Expression of the Salmonella enterica serovar Typhimurium Invasive Phenotype. Infect Immun **71:**1295-1305.

26. **Berg, G. G., Miles, E.F.** 1978. Binding of mercurials to membrane suspensions and undenatured proteins. Membr Biochem **2:**117-134.

27. **Blanc-Potard, A. B., Groisman, E.A.** 1997. The Salmonella selC Locus Contains a Pathogenicity Island Mediating Intramacrophage Survival. EMBO **16:**5376-5385.

28. **Blanc-Potard, A. B., Solomon, F., Kayser, J., Groisman, E.A.** 1999. The SPI-3 Pathogenicity Island of Salmonella enterica. J. Bacteriol. **181:**998-1004.

29. **Boucrot, E., Henry, T., Borg, J.P., Gorvel, J.P., Meresse, S.** 2005. The Intracellular Fate of Salmonella Depends on the Recruitment of Kinesin. Science **308:**1174-1178.

30. **Boyd, D., Peters, G.A., Cloeckaert, A., Boumedine, K.S., Chaslus-Dancla, E., Imberechts, H., Mulvey, M.R.** 2001. Complete Nucleotide Sequence of a 43-kilobase Genomic Island Associated with the Multidrug Resistance Region of Salmonella enterica serovar Typhimurium DT104 and its Identification in Phage Type DT120 and Serovar Agona. J. Bacteriol. **183:**5725-5732.

31. **Boyle, E. C., Brown, N.F., Finlay, B.B.** 2006. Salmonella enterica serovar Typhimurium Effectors SopB, SopE, SopE2 and SipA Disrupt Tight Junction Structure and Function. Cell Microbiol **8:**1946-1957.

32. **Braddock, M., Quinn, A., Canvin, J.** 2004. Therapeutic Potential of Targeting Il-1 and IL-18 in Inflammation. Expert Opin Biol Ther **4:**847-860.

33. **Brawn, L. C., Hayward, R.D., Koronakis, V.** 2007. Salmonella SPI1 Effector SipA Persists After Entry and Cooperates with a SPI2 Effector to Regulate Phagosome Maturation and Intracellular Replication. Cell Host & Microbe **1:**63-75.

34. **Brennan, M. A., Cookson, B.T.** 2000. Salmonella Induces Macrophage Death by Caspase-1-dependent Necrosis. Mol Microbiol **38:**31-40.

35. **Brenner, F. W., R. G. Villar, F. J. Angulo, R. Tauxe, and B. Swaminathan.** 2000. Salmonella Nomenclature. J. Clin. Microbiol. **38:**2465-2467.

36. **Bronstein, P. A., E. A. Miao, and S. I. Miller.** 2000. InvB Is a Type III Secretion Chaperone Specific for SspA. J. Bacteriol. **182:**6638-6644.

37. **Brown, L. L.** 1997. Evidence that the causal agent of bacterial cold-water disease *Flavobacterium psychrophilum* is transmitted within salmonid eggs. Dis Aquat Org **29:**213-218.

38. **Brown, N. F. e. a.** 2005. Salmonella Pathogenicity Island 2 is Expressed Prior to Penetrating the Intestine. PloS Pathog. **1**.

39. **Buchmeier, N., Bossie, S., Chen, C.Y., Fang, F.C., Guiney D.G., Libby, S.J.** 1997. SlyA, a Transcriptional Regulator of Salmonella typhimurium, is Required for Resistance to Oxidative Stress and is Expressed in the Intracellular Environment of Macrophages. Infect Immun **65:**3725-3730.

40. **Bullock, G. L., Snieszko, S.F.** 1975. Hagerman redmouth, a disease of salmonids caused by a member of the Enterobacteriaceae, p. 1-5. *In* F. a. W. S. United States Department of the Interior (ed.).

41. **Burr, S. E., K. Stuber, and J. Frey.** 2003. The ADP-Ribosylating Toxin, AexT, from Aeromonas salmonicida subsp. salmonicida Is Translocated via a Type III Secretion Pathway. J. Bacteriol. **185:**6583-6591.

42. **Burr, S. E., K. Stuber, T. Wahli, and J. Frey.** 2002. Evidence for a Type III Secretion System in Aeromonas salmonicida subsp. salmonicida. J. Bacteriol. **184:**5966-5970.

43. **Burr, S. E., Wahli, T., Segner, H., Pugovkin, D., Frey, J.** 2003. Association of Type III Secretion Genes with Virulence of *Aeromonas salmonicida* subsp. *salmonicida*. Dis Aquat Org **57:**167-171.

44. **Burrell, R.** 1993. Human Immune Toxicity. Mol Aspects Med **14:**1-81.

45. **Busch, R. A.** 1973. The serological surveillance of salmonid populations for presumptive evidence of specific disease association. University of Idaho.

46. **Busch, R. A., Lingg, A.** 1975. Establishment of an asymptomatic carrier state infection of enteric redmouth disease in rainbow trout (*Salmo gairdneri*). J. Fish. Res. Board Canada **32:**2429-2432.

47. **Cascales, E., Christie, P.J.** 2003. The Versatile Bacterial Type IV Secretion Systems. Nat. Rev. Microbiol. **1:**137-149.

48. **Cebrian, M. E., Albores, A., Aguilar, M., Blakely, E.** 1983. Chronic arsenic poisoning in the north of Mexico. Hum Toxicol **2:**121-133.

49. **Center for Genome Research and Biocomputing, O. S. U.**, posting date. Concentration of Template and Primer. [Online.]

50. **Chen, C., L. Eckmann, S. Libby, F. Fang, S. Okamoto, M. Kagnoff, J. Fierer, and D. Guiney.** 1996. Expression of Salmonella typhimurium rpoS and rpoS-dependent genes in the intracellular environment of eukaryotic cells. Infect. Immun. **64:**4739-4743.

51. **Chen, L. M., Kaniga, K., Galan, J.E.** 1996. Salmonella spp. are Cytotoxic for Culture Macrophages. Mol Microbiol **21:**1101-1115.

52. **Choi, M. H., Cech, J.J., Jr.** 1998. Unexpectedly high mercury level in pelleted commerical fish feed. Environ Toxicol Chem **17:**1979-1981.

53. **Cirillo, D. M. V., R.H., Monack, D.M., Falkow, S.** 1998. Macrophage-dependent Induction of the Salmonella Pathogenicity Island 2 Type III Secretion System and its role in Intracellular Survival. Mol Microbiol **30:**175-188.

54. **Clements, M., Eriksson, S., Tezcan-Merdol, D., Hinton, J.C.D., Rhen, M.** 2001. Virulence Gene Regulation in Salmonella enterica. Ann Med **33:**178-185.

55. **Coburn, B., Grassl, G.A., Finlay, B.B.** 2007. Salmonella the Host and Disease: a Brief Review. Immunol. Cell Biol. **85:**112-118.

56. **Collazo, C., Galan, J.E.** 1997. The Invasion-associated Type III Secretion System of Salmonella typhimurium Directs the Translocation of Sip Proteins into the Host Cell. Molecular Microbiology **24:**747-756.

57. **Collazo, C., Galan, J.E.** 1996. Requirement of Exported Proteins for Secretion Through the Invasion-associated Type III System in Salmonella typhimurium. Infect. Immun. **64:**3524-3531.

58. **Collier-Hyams, L. S. e. a.** 2002. Cutting Edge: Salmonella AvrA Effector Inhibits the Key Proinflammatory, Anti-apoptotic NF-kappaB Pathway. J Immunol **169:**2846-2850.

59. **Cone, D. K.** 1982. A *Lactobacillus* sp. from diseased female rainbow trout, *Salmo gairdneri* Richardson, in Newfoundland, Canada. J Fish Dis **5:**479-485.

60. **Coquet, L., P. Cosette, L. Quillet, F. Petit, G.-A. Junter, and T. Jouenne.** 2002. Occurrence and Phenotypic Characterization of Yersinia ruckeri Strains with Biofilm-Forming Capacity in a Rainbow Trout Farm. Appl. Environ. Microbiol. **68:**470-475.

61. **Crago, A., Koronakis, V.** 1998. Salmonella InvG Forms a Ring-like Multimer that Requires the InvH Lipoprotein for Outer Membrane Localization. Molecular Microbiology **30:**47-56.

62. **Crump, J. A., Luby, S.P., Mintz, E.D.** 2004. The Global Burden of Typhoid Fever. Bull. World Health Organ. **82:**346-353.

63. **Curtis, T., R. M. Z. G. Naal, C. Batt, J. Tabb, and D. Holowka.** 2008. Development of a mast cell-based biosensor. Biosensors and Bioelectronics **23:**1024-1031.

64. **Dacanay, A., S. C. Johnson, R. Bjornsdottir, R. O. Ebanks, N. W. Ross, M. Reith, R. K. Singh, J. Hiu, and L. L. Brown.** 2003. Molecular Characterization and Quantitative Analysis of Superoxide Dismutases in Virulent and Avirulent Strains of Aeromonas salmonicida subsp. salmonicida. J. Bacteriol. **185:**4336-4344.

65. **Dacanay, A., L. Knickle, K. S. Solanky, J. M. Boyd, J. A. Walter, L. L. Brown, S. C. Johnson, and M. Reith.** 2006. Contribution of the type III secretion system (TTSS) to virulence of Aeromonas salmonicida subsp. salmonicida. Microbiology **152:**1847-1856.

66. **Daefler, S., Russel, M.** 1998. The Salmonella typhimurium InvH Protein is an Outer Membrane Lipoprotein Required for the Proper Localization of InvG. Molecular Microbiology **28:**1367-1380.

67. **Darwin, K. H., Miller, V.L.** 1999. InvF is Required for Expression of Genes Encoding Proteins Secreted by the SPI-1 Type III Secretion Apparatus in Salmonella typhimurium. J Bacteriol **181:**4949-4954.

68. **De Boer, E., Beumer, R.R.** 1999. Methodology for Detection and Typing of Foodborne Microorganisms. Int J Food Microbiol **50:**119-130.

69. **Deiwick, J. e. a.** 1998. Mutations in Salmonella Pathogenicity Island 2 (SPI-2) Genes Affecting Transcription of SPI-1 Genes and Resistance to Antimicrobial Agents. J. Bacteriol. **180:**4775-4780.

70. **Desin, T. S., P.-K. S. Lam, B. Koch, C. Mickael, E. Berberov, A. L. S. Wisner, H. G. G. Townsend, A. A. Potter, and W. Koster.** 2009. Salmonella enterica Serovar Enteritidis Pathogenicity Island 1 Is Not Essential for but Facilitates Rapid Systemic Spread in Chickens. Infect. Immun. **77:**2866-2875.

71. **Dierksen, K. P., Mojovic, L., Caldwell, B.A., Preston, R.R., Upson, R., Lawrence, J., McFadden, P.N., Trempy, J.E.** 2004. Responses of Fish Chromatophore-based Cytosensor to a Broad Range of Biological Agents. Journal of Applied Toxicology **24:**363-369.

72. **Ding, Z., Atmakuri, K., Christie, P.J.** 2003. The outs and Ins of Bacterial Type IV Secretion Substrates. Trends in Microbiology **11:**527-535.

73. **Drechtrah, D., Knodler, L.A., Galbraith, K., Steele-Mortimer, O.** 2005. The Salmonella SPI1 Effector SopB Stimulates Nitric Oxide Production Long After Invasion. Cellular Microbiology **7:**105-113.

74. **Duchaud, E., M. Boussaha, V. Loux, J.-F. Bernardet, C. Michel, B. Kerouault, S. Mondot, P. Nicolas, R. Bossy, C. Caron, P. Bessieres, J.-F. Gibrat, S. Claverol, F. Dumetz, M. L. Henaff, and A. Benmansour.** 2007. Complete genome sequence of the fish pathogen Flavobacterium psychrophilum. Nat Biotech **25:**763-769.

75. **Dukes, J. D., H. Lee, R. Hagen, B. J. Reaves, A. N. Layton, E. E. Galyov, and P. Whitley.** 2006. The secreted Salmonella dublin phosphoinositide phosphatase, SopB, localizes to PtdIns(3)P-containing endosomes and perturbs normal endosome to lysosome trafficking. Biochem J **395:**239-247.

76. **Ebanks, R. O., A. Dacanay, M. Goguen, D. M. Pinto, and N. W. Ross.** 2004. Differential proteomic analysis of <B><I>Aeromonas salmonicida</I></B> outer membrane proteins in response to low iron and <B><I>in vivo</I></B> growth conditions. PROTEOMICS **4:**1074-1085.

77. **Ehrbar, K., A. Friebel, S. I. Miller, and W.-D. Hardt.** 2003. Role of the Salmonella Pathogenicity Island 1 (SPI-1) Protein InvB in Type III Secretion of SopE and SopE2, Two Salmonella Effector Proteins Encoded Outside of SPI-1. J. Bacteriol. **185:**6950-6967.

78. **Eichelberg, K., Galan, J.E.** 1999. Differential Regulation of Salmonella typhimurium Type III Secreted Proteins by Pathogenicity Island 1 (SPI-1)-encoded Transcriptional Activators InvF and HilA. Infect Immun **67:**4099-4105.

79. **Ellenhorn, M. J., Schonwald, S., Ordog, G., Wasserberger, J.** 1997. Metals and related compounds, p. 1589-1599, Ellenhorn's medical toxicology: Diagnosis and treatment of human poisoning. Williams & Wilkins, Baltimore.

80. **Ellermeier, C. D., Ellermeier, J.R., Slauch, J.M.** 2005. HilD, HilC and RtsA Constitute a Feed Forward Loop that Controls Expression of the SPI-1 Type III Secretion System Regulator HilA in Salmonella enterica serovar Typhimurium. Mol Microbiol **57:**691-705.

81. **Ellermeier, C. D., Slauch, J.M.** 2003. RtsA and RtsB Coordinately Regulate Expression of the Invasion and Flagellar Genes in Salmonella enterica serovar Typhimurium. J Bacteriol **185:**5096-5108.

82. **Ellermeier, J. R., and J. M. Slauch.** 2007. Adaptation to the host environment: regulation of the SPI1 type III secretion system in Salmonella enterica serovar Typhimurium. Current Opinion in Microbiology **10:**24-29.

83. **Ellermeier, J. R., and J. M. Slauch.** 2008. Fur Regulates Expression of the Salmonella Pathogenicity Island 1 Type III Secretion System through HilD. J. Bacteriol. **190:**476-486.

84. **Espina, M., Olive, A., Kenjale, R., Moore, D., Ausar, S., Kaminski, R., Oaks, E., Middaugh, C., Picking, W., Picking, W.** 2006. IpaD Localizes to the Tip of the Type III Secretion System Needle of Shigella flexneri. Infect. Immun. **74:**4391-4400.

85. **Fang, F., Libby, SJ, Buchmeier, NA, Loewen, PC, Switala, J, Harwood, J, Guiney, DG.** 1992. The Alternative Sigma FActor katF (rpoS) Regulates Salmonella Virulence. Proc Natl Acad Sci USA **89:**11978-11982.

86. **Feng, X., Oropenza, R., Kenney, L.J.** 2003. Dual Regulation by Phospho-OmpR of ssrA/B Gene Expression in Salmonella Pathogenicity Island 2. Mol Microbiol **48:**1131-1143.

87. **Fernandez, L., J. R. Lopez, P. Secades, A. Menendez, I. Marquez, and J. A. Guijarro.** 2003. In Vitro and In Vivo Studies of the Yrp1 Protease from Yersinia ruckeri and Its Role in Protective Immunity against Enteric Red Mouth Disease of Salmonids. Appl. Environ. Microbiol. **69:**7328-7335.

88. **Fernandez, L., I. Marquez, and J. A. Guijarro.** 2004. Identification of Specific In Vivo-Induced (ivi) Genes in Yersinia ruckeri and Analysis of Ruckerbactin, a Catecholate Siderophore Iron Acquisition System. Appl. Environ. Microbiol. **70:**5199-5207.

89. **Fernandez, L., M. Prieto, and J. A. Guijarro.** 2007. The iron- and temperature-regulated haemolysin YhlA is a virulence factor of Yersinia ruckeri. Microbiology **153:**483-489.

90. **Fitzpatrick J., F., L., Hearty, S., Leonard, P., Manning, B.M., Quinn, J.G. et al.** 2000. Applications and Recent Developments in the use of Antibodies for Analysis. Anal Lett **33:**263-2609.

91. **Folkesson, A., Lofdahl, S., Normark, S.** 2002. The Salmonella enterica Subspecies I Specific Centisome 7 Genomic Island Encodes Novel Protein Families Present in Bacteria Living in Close Contact with Eukaryotic Cells. Res. Microbiol. **153:**537-545.

92. **Franchi, L., Amer, A., Body-Malapel, M., Kanneganti, T.D., Ozoren, N., Jagirdar, R. et al.** 2006. Cytosolic Flagellin Requires Ipaf for Activation of Caspase-1 and Interleukin-1beta in Salmonella-infected Macrophages. Nat Immunol **7:**576-582.

93. **Frenzen, P. D., Riggs, T.L., Buzby, J.C. et al.** 1999, posting date. Salmonella Cost Estimate Updated Using FoodNet Data. [Online.]

94. **Friberg, L., Enestrom, S.** 1990. Toxicology of inorganic mercury, p. 163-173. *In* A. D. Dayan, Hertel, R.F., Heseltine, E., Kazantzis, G., Smith, E.M., Van der Venne, M.T. (ed.), Immunotoxicity of metals and immunotoxicology. Plenum Press, New York.

95. **Friebel, A., H. Ilchmann, M. Aepfelbacher, K. Ehrbar, W. Machleidt, and W.-D. Hardt.** 2001. SopE and SopE2 from Salmonella typhimurium Activate Different Sets of RhoGTPases of the Host Cell. J. Biol. Chem. **276:**34035-34040.

96. **Fu, Y., Galan, J.E.** 1999. A Salmonella Protein Antagonizes Rac-1 and Cdc42 to Mediate Host-cell Recovery After Bacterial Invasion. Nature **401:**293-297.

97. **Fu, Y., Galan, J.E.** 1998. The Salmonella typhimurium Tyrosine Phosphatase SptP is Translocated into Host Cells and Disrupts the Actin cytoskeleton. Mol Microbiol **27:**359-368.

98. **Fuhrmann, H., Bohm, K.H., Schlotfeldt, H.-J.** 1983. An outbreak of enteric redmouth disease in West Germany. Journal of Fish Diseases **6:**309-311.

99. **Fujii, R.** 1993. Cytyophysiology of Fish Chromatophores. International Review of Cytology **143:**191-255.

100. **Galán, J. E.** 1996. Molecular and Cellular Bases of *Salmonella* Entry Into Host Cells. Curr Top Microbiol Immunol **209:**43-60.

101. **Gallagher, E. a. B., C.** 2007. Residual Chlorine Levels in Municipal Drinking Water. *In* S. R. Dukovcic (ed.). Pacific Analytical Laboratory, Corvallis.

102. **Garduno, R. A., Kuzyk, M.A., Kay, W.W.** 1997. Structural and Physiological Determinants of Resistance of Aeromonas salmonicida to Reactive Radicals. Can J Microbiol **43:**1044-1053.

103. **Garner, M. J., Hayward, R.D., Koronakis, V.** 2002. The Salmonella Pathogenicity Island 1 Secretion System Directes Cellular Cholesterol Redistribution During Mammalian Cell Entry and Intracellular Trafficking. Cellular Microbiology **4:**153-165.

104. **Gerlach, R. G., and M. Hensel.** 2007. Protein secretion systems and adhesins: The molecular armory of Gram-negative pathogens. International Journal of Medical Microbiology **297:**401-415.

105. **Giacomodonato, M. N. e. a.** 2007. SipA, SopA, SopB, SopD and SopE2 Effector Proteins of Salmonella enterica serovar Typhimurium are synthesized at Late Stages of Infection in Mice. Microbiology **153:**1221-1228.

106. **Goley, E. D., Welch, M.D.** 2006. The ARP2/3 Complex: an Actin Nucleator Comes of Age. Nat Rev Mol Cell Biol **7:**713-726.

107. **Goodrich, H. B., and R. N. Mercer.** 1934. Genetics and Colors of the Siamese Fighting Fish, Betta splendens. Science **79:**318.

108. **Group, T. R.** 2007. Year 2005 and 2006 Review and Year 2007 Outlook. Oregon Departmen of Fish and Wildlife, Oregon Coastal Zone Management Association Inc.

109. **Gu, H., K. Xu, C. Xu, and B. Xu.** 2006. Biofunctional magnetic nanoparticles for protein separation and pathogen detection. Chemical Communications**:**941-949.

110. **Gunasena, D. K., Komrower, J.R., Macintyre, S.** 2003. The Fish Pathogen Yersinia ruckeri Possesses a TTS system, p. 105-107. *In* M. Skurnik, Benoechea, J.A., Granfors, K. (ed.), The Genus Yersinia: Entering the Functional Genomic Era. Kluwer Academic/Plenum, New York.

111. **Handy, R. D., Poxton, M.G.** 1993. Nitrogen Pollution in Mariculture: Toxicity and Excretion of Nitrogenous Compounds by Marine Fish. Rev. Fish Biol. Fish. **3:**205-241.

112. **Hansen-Wester, I., and M. Hensel.** 2001. Salmonella pathogenicity islands encoding type III secretion systems. Microbes and Infection **3:**549-559.

113. **Hansen-Wester, I., Hensel, M.** 2002. Genome-based Identification of Chromosomal Regions Specific for Salmonella spp. Infect. Immun. **70:**2351-2360.

114. **Haraga, A., Miller, S.I.** 2003. A Salmonella enterica serovar Typhimurium Translocated Leucine-rich Repeat Effector Protein Inhibits NF-kappaB-dependent Gene Expression. Infect. Immun. **71:**4052-4058.

115. **Haraga, A., Miller, S.I.** 2006. A Salmonella Type III Secretion Effector Interacts with the Mammalian Serine/Threonine Protein Kinase PKN1. Cell. Microbiol. **8:**837-846.

116. **Haraga, A., M. B. Ohlson, and S. I. Miller.** 2008. Salmonellae interplay with host cells. Nat Rev Micro **6:**53-66.

117. **Hardt, W.-D., L.-M. Chen, K. E. Schuebel, X. R. Bustelo, and J. E. Galán.** 1998. S. typhimurium Encodes an Activator of Rho GTPases that Induces Membrane Ruffling and Nuclear Responses in Host Cells. Cell **93:**815-826.

118. **Hayward, R. D., Cain, R.J., McGhie, E.J., Phillips, N., Garner, M.J., Koronakis, V.** 2005. Cholesterol Binding by the Bacterial Type III Translocon is Essential for Virulence Effector Delivery into Mammalian Cells. Molecular Microbiology **56:**590-603.

119. **Hayward, R. D., Koronakis, V.** 1999. Direct Nucleation and Bundling of Actin by the SipC Protein of Invasive Salmonella. EMBO **18:**4926-4934.

120. **Hensel, M.** 2004. Evolution of pathogenicity islands of Salmonella enterica. International Journal of Medical Microbiology **294:**95-102.

121. **Hensel, M. e. a.** 1997. Functional Analysis of ssaJ and the ssaK/U Operon, 13 Genes Encoding Components of the Type III Secretion Apparatus of Salmonella Pathogenicity Island 2. Molecular Microbiology **24:**155-167.

122. **Hernandez, L. D., Hueffer, K., Wenk, M.R., Galan, J.E.** 2004. Salmonella modulates vesicular traffic by Altering Phosphoinositide Metabolism. Science **304:**1805-1807.

123. **Hernandez, L. D., Pypaert, M., Flavell, R.A., Galan, J.E.** 2003. A Salmonella Protein Causes Macrophage Cell Death by Inducing Autophagy. J.Cell Biol. **163:**1123-1131.

124. **Hersh, D. e. a.** 1999. The Salmonella Invasin SipB Induces Macrophage Apoptosis by Binding to Caspase-1. Proceedings of the National Accademy of Sciences **96:**2396-2401.

125. **Hester, F. E.** 1973. Fish health: a nationwide survey of problems and needs. Progressive Fish Culturist **35:**11-18.

126. **Hogben, L. T., Winton, F.R.** 1922. The Pigmentary Effector System: Reaction of Frog's Melanophores to Pituitary Extracts. Proc Roy Soc **B93:**318-329.

127. **Holmstrom, A., Olsson, J., Cherepanov, P., Maier, E., Nordfelth, R., Pettersson, J. et al.** 2001. LcrV is a Channel Size-determining Component of the Yop Effector Translocon of Yersinia. Molecular Microbiology **39:**620-632.

128. **Hu, Q., B. Coburn, W. Deng, Y. Li, X. Shi, Q. Lan, B. Wang, B. K. Coombes, and B. B. Finlay.** 2008. Salmonella enterica Serovar Senftenberg Human Clinical Isolates Lacking SPI-1. J. Clin. Microbiol. **46:**1330-1336.

129. **Hughes, M. F.** 2002. Arsenic toxicity and potential mechanisms of action. Toxicology Letters **133:**1-16.

130. **Hui, S. F., Holt, R.A., Sriranganathan, N., Seidler, R.J., Fryer, J.L.** 1984. *Lactobacillus piscicola*, a new species from salmonid fish. Int J Sys Bacter **34:**393-400.

131. **Humphreys, S., A. Stevenson, A. Bacon, A. B. Weinhardt, and M. Roberts.** 1999. The Alternative Sigma Factor, sigma E, Is Critically Important for the Virulence of Salmonella typhimurium. Infect. Immun. **67:**1560-1568.

132. **Hunter, V. A., Knittel, M.D., Fryer, J.L.** 1980. Stress-induced transmission of *Yersinia ruckeri* infection from carriers to recipient steelhead trout *Salmo gairdneri*. J Fish Dis **3:**467-472.

133. **Hutchison, J. R.** 2009. Bacterial Pathogens and Associated Toxins Involved in Erythrophore Cell Aggregation. Oregon State University, Corvallis.

134. **Hutchison, J. R., Dukovcic, S.R., Dierksen, K.P., Carlyle, C.A., Caldwell, B.A., Trempy, J.E.** 2008. Erythrophore Cell Response to Food-associated Pathogenic Bacteria: Implications for Detection. Microbial Biotechnology **1:**425-431.

135. **Iel-Soo Bang, Jonathan G. Frye, Michael McClelland, Jyoti Velayudhan, and Ferric C. Fang.** 2005. Alternative sigma factor interactions in Salmonella: σ<sup>E</sup> and σ<sup>H</sup> promote antioxidant defences by enhancing σ<sup>S</sup> levels. Molecular Microbiology **56:**811-823.

136. **Iga, T., Takabatake, I.** 1982. Action of melanophore-stimulating hormone on melanophores of the cyprinid fish *Zacco temmincki*. Comp. Biochem. Physiol. **73C:**51-55.

137. **Invitski, D., Abdel-Hamid, I., Atanasov, P, Wilkins, E.** 1999. Biosensors for the Detection of Pathogenic Bacteria. Biosensors and Bioelectronics **14:**599-624.

138. **Ip, Y. K., Chew, S.F., Randall, D.J.** 2001. Ammonia Toxicity, Tolerance, and Excretion, p. 109-148. *In* P. A. Wright, Anderson, P.M. (ed.), Nitrogen Excretion, vol. 20. Academic Press.

139. **Janeway, C. A., Travers, P., Walport, M., Shlomchik, M.J.** 2005. Immunobiology: the immune system in health and disease, 6 ed. Garland Science Publishing, New York.

140. **Jesenberger, V., Procyk, K.J., Yuan, J., Reipert, S., Baccarini, M.** 2000. Salmonella-induced Caspase-2 Activation in Macrophages: a Novel Mechanism in Pathogen-mediated Apoptosis. J Exp Med **192:**1035-1046.

141. **Johnson, L. L., Casilla, E, Collier, J.K., McCain, B.B., Varanasi, U.** 1988. Contaminant effects on ovarian development in English sole (*Parophrys vetulus*) from Pugent Sound, Washington. Can J Fish Aquat Sci **45:**2133-2146.

142. **Jones, B. D., Ghori, N., Falkow, S.** 1994. Salmonella typhimurium Initiates Murine Infection by Penetrating and Destroying the Specialized Epithelial M Cells of the Peyer's Patches. J. Exp. Med. **180:**15-23.

143. **Jones, R. M., H. Wu, C. Wentworth, L. Luo, L. Collier-Hyams, and A. S. Neish.** 2008. Salmonella AvrA Coordinates Suppression of Host Immune and Apoptotic Defenses via JNK Pathway Blockade. Cell Host & Microbe **3:**233-244.

144. **Kaniga, K., Uralil, J., Bliska, J.B., Galan, J.E.** 1996. A Secreted Protein Tyrosine Phosphatase with Modular Effector Domains in the Bacterial Pathogen Salmonella typhimurium. Mol Microbiol **21:**633-641.

145. **Karlsson, A. M., K. Bjuhr, M. Testorf, P. Oberg, E. Lerner, I. Lundstrom, and S. P. S. Svensson.** 2002. Biosensing of opioids using frog melanophores. Biosensors and Bioelectronics **17:**331-335.

146. **Kennedy, M., Villar, R., Vugia, D.J., Rabatsky-Ehr, T., Farley, M.M., Pass, M., et al.** 2004. Hospitalizations and Dealths Due to Salmonella Infections, FoodNet, 1996-1999. Clin. Infect. Dis. **38:**S142-S148.

147. **Khan, N. A., Deschaux, P.** 1997. Role of Serotonin in Fish Immunomodulation. The Journal of Experimental Biology **200:**1833-1383.

148. **Khan, N. A., Troutaud, D., Deschaux, P.** 1996. Characterization of Serotonin Receptors in Fish Brain: Modulation by Polyamines. Neuroscience Research Communications **18:**97-105.

149. **Kim, J. M., Eckmann, L., Savidge, T.C., Lowe, D.C., Witthoft, T., Kagnoff, M.F.** 1998. Apoptosis of Human Intestinal Epithelial Cells After Bacterial Invasion. J Clin Invest **102:**1815-1823.

150. **Kimbrough, T. G., and S. I. Miller.** 2002. Assembly of the type III secretion needle complex of Salmonella typhimurium. Microbes and Infection **4:**75-82.

151. **Kimbrough, T. G., Miller, S.I.** 2000. Contribution of Salmonella typhimurium Type III Secretion Components to Needle Complex Formation. Proceedings of the National Accademy of Sciences **97:**11008-11013.

152. **Kinzler, K. W., Vogelstein, B.** 1996. Lessons from Hereditary Colorectal Cancer. Cell **87:**159-170.

153. **Klontz, G. W., Huddleston, T.R.** 1976. Control of Enteric Redmouth Disease. University of Idaho.

154. **Kohbata, S., Yokoyama, H., Yabuuchi, E.** 1986. Cytopathogenic Effect of Salmonella typhi GIFU 10007 on M Cells of Murine Ileal Peyer's Patches in Ligated Ileal Loops: an Ultrastructural Study. Microbiol. Immunol. **30:**1225-1237.

155. **Koronakis, V., Koronakis, E., Hughes, C.** 1989. Isolation and Analysis of the C-terminal Signal Directing Export of Escherichia coli Hemolysin Protein Across Both Bcterial Membranes. EMBO **8:**595-605.

156. **Kostakioti, M., Newman, C.L., Thanassi, D.G., Stathopoulos, C.** 2005. Mechanisms of Protein Export Across the Bacterial Outer Membrane. Journal of Bacteriology **187:**4306-4314.

157. **Kostyniak, P. J.** 1991. Mechanisms of urinary excretion of methylmercury, p. 99-109. *In* T. Suzuki, Imura, N., Clarkson, T.W. (ed.), Advances in mercury toxicology. Plenum Press, New York.

158. **Kubori, T., Galan, J.E.** 2003. Temporal Regulation of Salmonella Virulence Effector Function by Proteasome-dependent Protein Degradation. Cell **115:**333-342.

159. **Kubori, T., Sukhan, A., Aizawa, S.I., Galan, J.E.** 2000. Molecular Characterization and Assembly of the Needle Complex of the Salmonella typhimurium Type III Protein Secretion System. Proceedings of the National Accademy of Sciences **97:**10225-10230.

160. **Kubori, T. e. a.** 1998. Supramolecular Structure of the Salmonella typhimurium Type III Secretion System. Science **280:**602-605.

161. **Lafont, F., Tran Van Nhieu, G., Hanada, K., Sansonetti, P.J., van der Goot, F.G.** 2002. Initial Steps of Shigella Infection Depend on the Cholesterol/Spingolipid Raft-mediated CD44-IpaB Interaction. EMBO **21:**4449-4457.

162. **Land, E. J., Ramsden, C.A., Riley, P.A.** 2006. Toxicological aspects of melanin and melanogenesis, p. 354-394. *In* J. J. Nordlund, Boissy, R.E., Hearing, V.J., King, R.A., Ortonne, J.P. (ed.), The Pigmentary System. Oxford University Press, Oxford.

163. **Lara-Tejero, M., and J. E. Galan.** 2009. Salmonella enterica Serovar Typhimurium Pathogenicity Island 1-Encoded Type III Secretion System Translocases Mediate Intimate Attachment to Nonphagocytic Cells. Infect. Immun. **77:**2635-2642.

164. **Larsson, B.** 1993. Interaction between chemicals and melanin. Pigment Cell Research **6:**127-133.

165. **Lawhon, S. D., Maurer, R., Suyemoto, M., Altier, C.** 2002. Intestinal Short-chain Fatty Acids Alter Salmonella typhimurium Invasion Gene Expression and Virulence through BarA/SirA. Mol Microbiol **46:**1451-1464.

166. **Lawley, T. D. e. a.** 2006. Genome-wide Screen for Salmonella Genes Required for Long-term Systemic Infection of the Mouse. PloS Pathog. **2**.

167. **Layton, A. N., Galyov, E.E.** 2007. Salmonella-induced Enteritis: Molecular Pathogenesis and Therapeutic Implications. Expert Rev Mol Med **9:**1-17.

168. **Lazcka, O., F. J. D. Campo, and F. X. Muñoz.** 2007. Pathogen detection: A perspective of traditional methods and biosensors. Biosensors and Bioelectronics **22:**1205-1217.

169. **Lee-Feldstein, A.** 1983. Arsenic and respiratory cancer in human: follow-up of copper smelter employees in Montana. J Natl Cancer Inst **70:**601-609.

170. **Lee-Feldstein, A.** 1986. Cumulative exposure to arsenic and its relationship to respiratory cancer among copper smelter employees. J Occup Med **28:**296-302.

171. **Lehmann, J., Mock, D., Sturenber, F.-J., Bernardet, J.-F.** 1991. First isolation of *Cytophaga psychrophila* from a systemic disease in eel and cyprinids. Dis Aquat Org **10:**217-220.

172. **Leonard, P., S. Hearty, J. Brennan, L. Dunne, J. Quinn, T. Chakraborty, and R. O'Kennedy.** 2003. Advances in biosensors for detection of pathogens in food and water. Enzyme and Microbial Technology **32:**3-13.

173. **Liao, A. P., E. O. Petrof, S. Kuppireddi, Y. Zhao, Y. Xia, E. C. Claud, and J. Sun.** 2008. <italic>Salmonella</italic> Type III Effector AvrA Stabilizes Cell Tight Junctions to Inhibit Inflammation in Intestinal Epithelial Cells. PLoS ONE **3:**e2369.

174. **Lister, J.** 1858. On the Cutaneous Pigmentary System of the Frog. Philos Trans **148:**627-643.

175. **Llosa, M., O'Callaghan, D.** 2004. Euroconference on the Biology of Type IV Secretion Processes: Bacterial Gates into the Outer World. Mol Microbiol **53:**1-8.

176. **Loferer, H., Hammar, M., Normark, S.** 1997. Availability of the Fibre Subunit CsgA and the Nucleator Protein CsgB During Assembly of Fibronectin-binding Curli is Limited by the Intracellular Concentration of the Novel Lipoprotein CsgG. Mol Microbiol **26:**11-23.

177. **Lostroh, C. P., and C. A. Lee.** 2001. The HilA Box and Sequences outside It Determine the Magnitude of HilA-Dependent Activation of PprgH from Salmonella Pathogenicity Island 1. J. Bacteriol. **183:**4876-4885.

178. **Marlovits, T. C. e. a.** 2006. Assembly of the Inner Rod Determines Needle Length in the Type III Secretion Injectisome. Nature **441**.

179. **Marlovits, T. C. e. a.** 2004. Structural Insights into the Assembly of the Type III Secretion Needle Complex. Science **306:**1040-1042.

180. **Marsh, M. C.** 1902. *Bacterium truttae,* a new bacterium pathogenic to trout. Science **16:**706.

181. **Maurer-Spurej, E.** 2005. Circulating Serotonin in Vertebrates. Cellular and Molecular Life Sciences **62:**1881-1889.

182. **McArdle, J. F., Dooley-Martin, C.** 1985. Isolation of *Yersinia ruckeri* type 1 (Hagerman strain) from goldfish *Carassius auratus* (L.). Bull. Euro. Assoc. Fish Path. **5:**10-11.

183. **McClelland, M., K. E. Sanderson, J. Spieth, S. W. Clifton, P. Latreille, L. Courtney, S. Porwollik, J. Ali, M. Dante, F. Du, S. Hou, D. Layman, S. Leonard, C. Nguyen, K. Scott, A. Holmes, N. Grewal, E. Mulvaney, E. Ryan, H. Sun, L. Florea, W. Miller, T. Stoneking, M. Nhan, R. Waterston, and R. K. Wilson.** 2001. Complete genome sequence of Salmonella enterica serovar Typhimurium LT2. Nature **413:**852-856.

184. **McCormick, B. A., Miller, S.I., Carnes, D., Madara, J.L.** 1995. Transepithelial Signaling to Neutrophils by Salmonellae: a Novel Virulence Mechanism for Gastroenteritis. Infect. Immun. **63:**2302-2309.

185. **McCormick, B. A. e. a.** 1995. Surface Attachment of Salmonella typhimurium to Intestinal Epithelia Imprints the Subepithelial Matrix with Gradients of Chemotactic for Neutrophils. J. Cell Biol. **131:**1599-1608.

186. **McDaniel, D. W.** 1972. Hatchery Biologist, Quarterly Report.

187. **Mead, P. S., Slutsker, L., Dietz, V., McCaig, L.F., Bresee, J.S., Chapiro, C., Griffin, P.M., Tauxe, R.V.** 1999. Food-related Illness and Death in the United States. Emerg Infect Dis **5:**607-625.

188. **Merighi, M., D. Majerczak, M. Zianni, K. Tessanne, and D. Coplin.** 2006. Molecular characterization of Pantoea stewartii subsp. stewartii HrpY, a conserved response regulator of the Hrp type III secretion system, and its interaction with the hrpS promoter. J Bacteriol **188:**5089 - 5100.

189. **Merighi, M., A. Septer, A. Carroll-Portillo, A. Bhatiya, S. Porwollik, M. McClelland, and J. Gunn.** 2009. Genome-wide analysis of the PreA/PreB (QseB/QseC) regulon of Salmonella enterica serovar Typhimurium. BMC Microbiology **9:**42.

190. **Miao, E. A., Alpuche-Aranda, C.M., Dors, M., Clark, A.E., Bader, M.W., Miller, S.I., Aderem, A.** 2006. Cytoplasmic Flagellin Activates Caspase-1 and Secretion of Interleukin-1beta via Ipaf. Nat Immunol **7:**569-575.

191. **Miao, E. A. e. a.** 1999. Salmonella typhimurium Leucine-rich Repeat Proteins are Targeted to the SPI1 and SPI2 Type III Secretion Systems. Mol. Microbiol **34:**850-864.

192. **Mirold, S., Ehrbar, K., Weissmuller, A., Prager, R., Tschape, H., Russmann, H., Hardt, W.D.** 2001. Salmonella Host Cell Invasion Emerged by Acquisition of a Mosaic of Separate Genetic Elements, Including Salmonella Pathogenicity Island 1 (SPI-1), SPI-5, and sopE2. J. Bacteriol. **183:**2348-2358.

193. **Mirold, S., Rabsch, W., Rohde, M., Stender, S., Tschape, H., Russmann, H., Igwe, E., Hardt, W.D.** 1999. Isolation of a Temperate Bacteriophage Encoding the Type III Effector Protein SopE from an Epidemic Salmonella typhimurium Strain. Proceedings of the National Accademy of Sciences **96:**9845-9850.

194. **Mojovic, L., Dierksen, K.P., Upson, R.H., Caldwell, B.A., Lawrence, J.R., Trempy, J.E., McFadden, P.N.** 2004. Blind and Naive Classification of Toxicity by Fish Chromatophores. Journal of Applied Toxicology **24:**355-361.

195. **Monack, D. M., Raupach, B., Hromockyj, A.E., Falkow, S.** 1996. Salmonella typhimurium Invasion Induces Apoptosis in Infected Macrophages. Proceedings of the National Accademy of Sciences **93:**9833-9838.

196. **Montville, T. J., Matthews, K.R.** 2005. Salmonella Species, p. 85-99, Food Microbiology, an Introduction. ASM Press, Washington DC.

197. **Moon, R. T., Bowerman, B., Boutros, M., Perrimon, N.** 2002. The Promise and Perils of Wnt Signaling Through Beta-catenin. Science **296:**1644-1646.

198. **Mouslim, C., Groisman, E.A.** 2003. Control of the Salmonella ugd gene by Three Two-component Regulatory Systems. Mol Microbiol **47:**335-344.

199. **Mullis, K., Faloona, F., Scharf, S., Saiki, R., Horn, G., Erlich, H.** 1986. Presented at the Cold Spring Harbor Symposia on Quantitative Biology.

200. **Murli, S., Watson, R.O., Galan, J.E.** 2001. Role of Tyrosine Kinases and the Tyrosine Phosphatase SptP in the Interaction of Salmonella with Host Cells. Cell Microbiol **3:**795-810.

201. **Narahashi, T.** 1996. Effects of metals on ion channels, p. 677-698. *In* L. W. Chang (ed.), Toxicology of metals. CRC Press, Boca Raton.

202. **Nickerson, C., and R. Curtiss, 3rd.** 1997. Role of sigma factor RpoS in initial stages of Salmonella typhimurium infection. Infect. Immun. **65:**1814-1823.

203. **Niimi, A. J., Kissoon, G.P.** 1994. Evaluation of the critical body burden concept based on inorganic and organic mercury toxicity to rainbow trout (*Oncorhynchus mykiss*). Arch Environ Contam Toxicol **26:**169-178.

204. **O'Callaghan, D., and A. Charbit.** 1990. High efficiency transformation of Salmonella typhimurium and Salmonella typhi by electroporation. Molecular and General Genetics MGG **223:**156-158.

205. **Oelschlaeger, T. A., Zhang, D., Schubert, S., Carniel, E., Rabsch, W., Karch, H., Hacker, J.** 2003. The High-pathogencitity Island is Absent in Human Pathogens of Salmonella enterica subspecies I but Present in Isolates of Subspecies III and VI. J. Bacteriol. **185:**1107-1111.

206. **Olekhnovich, I. N., Kadner R.J.** 2002. DNA-binding Activities of the HilC and HilD Virulence Regulatory Proteins of Salmonella enterica serovar Typhimurium. J Bacteriol **184:**4148-4160.

207. **Paesold, G., Guiney, D.G., Eckmann, L., Kagnoff, M.F.** 2002. Genes in the Salmonella Pathogenicity Island 2 and the Salmonella Virulence Plasmid are Essential for Salmonella-induced Apoptosis in Intestinal Epithelial Cells. Cell Microbiol **4:**771-781.

208. **Parkhill, J. e. a.** 2001. Complete Genome Sequence of a Multiple Drug Resistant Salmonella enterica serovar Typhi CT18. Nature **413:**848-852.

209. **Patel, J. C., Galan, J.E.** 2006. Differential Activation and Function of Rho GTPases During Salmonella-host Cell Interactions. J. Cell Biol. **175:**453-463.

210. **Patel, J. C., Galan, J.E.** 2005. Manipulation of the Host Actin Cytoskeleton by Salmonella - All in the Name of Entry. Curr Opin Microbiol **8:**10-15.

211. **Peabody, C. R., Chung, Y.J., Yen, M.R., Vidal-Ingigliardi, D., Pugsley, A.P., Saier, M.H. Jr.** 2003. Type II Protein Secretion and its Relationship to Bacterial Type IV Pili and Archaeal Flagella. Microbiology **149:**3051-3072.

212. **Polakis, P.** 2000. Wnt Signaling and Cancer. Genes Dev. **14:**1837-1851.

213. **Potenza, M. N., Lerner, M.R.** 1992. A rapid quantitative bioassay for evaluating the effects of ligands upon receptors that modulate cAMP levels in a melanophore cell line. Pigment Cell Research **5:**372-378.

214. **Prost, L., and S. Miller.** 2008. The Salmonellae PhoQ sensor: mechanisms of detection of phagosome signals. Cell Microbiol **10:**576 - 582.

215. **Rajan, M. T., Banerjee, T.K.** 1995. Melanophore Indexing: A New Bio-assay Technique for the Analysis of Acute Heavy Metal Toxicity. Biomedical and Environmental Sciences **8:**226-231.

216. **Randall, D. J., Wicks, B.J.** 2000. Presented at the Fifth International Symposium on Fish Physiology, Toxicology and Water Quality, Hong Kong, November 10-13, 1998.

217. **Rasooly, A.** 2006. Biosensors for the Analysis of Food- and Waterborne Pathgoens and Their Toxins. Journal of AOAC International **89:**873-883.

218. **Reddel, R. R.** 1995. Immortalization techniques. Methods in Cell Science **17:**65-66.

219. **Renfro, J. L., Schmidt-Nielsen, B., Miller, D., Benos, D., Allen, J.** 1974. Methylmercury and inorganic mercury: Uptake, distribution and effect on osmoregulatory mechanisms in fishes, p. 101-122. *In* F. J. Vernberg, Vernberg, W.B. (ed.), Polution and physiology of marine organisms. Academic Press, New York.

220. **Roberts, R. J.** 1975. Melanin Containing Cells of Teleost Fish and Their Relation to Disease, p. 399-428. *In* W. E. Ribelin, Migaki, G. (ed.), The Pathology of Fishes. The University of Wisconsin Press, Madison.

221. **Rodionov, V., Yi, J., Kashina, A., Oladipo, A., Gross, S.P.** 2003. Switching between microtubule- and actin-based transport systems in melanophores is controlled by cAMP levels. Curr Biol **13:**1837-1847.

222. **Rohde, J. R., Breitkreutz, A., Chenal, A., Sansonetti, P.J., Parsot, C.** 2007. Type III Secretion Effectors of the IpaH Family are E3 Ubiquitin Ligases. Cell Host & Microbe **1:**77-83.

223. **Romalde, J. L., Barja, J.L., Magarinos, B., Toranzo, A.E.** 1994. Starvation-survival processes of the bacterial fish pathogen *Yersinia ruckeri*. Sys. App. Microbiol. **17:**161-168.

224. **Ross, A. J., Toth, R.J.** 1974. *Lactobacillus* - a new fish pathogen? Progressive Fish Culturist **36:**191.

225. **Rozanowska, M., Sarna, T., Land, E.J., Truscott, TG. .** 1999. Free radical scavenging properties of melanin interaction of eu- and pheo-melanin models with reducing and oxidising radicals. Free Radic. Biol. Med. **26:**518-525.

226. **Rucker, R. R.** 1966. Redmouth disease of rainbow trout (*Salmo gairdneri*), p. 825-830. *In* L. O. I. d. Epizooties (ed.), vol. 65.

227. **Rucker, R. R., Earp, B.J., Ordal, E.J.** 1953. Infectious diseases of Pacific salmon. Trans. Amer. Fish. Society **83:**297-312.

228. **RYOZO FUJII*.** 2000. The Regulation of Motile Activity in Fish Chromatophores. Pigment Cell Research **13:**300-319.

229. **Salte, R., Norberg, K., Arnesen, J.A., Oedegaard, O.R., Eggset, G.** 1992. Serine Protease and Glycerophospholipid:cholsterol Acyltransferase of Aeromonas salmonicida Work in Concert in Thrombus Formation; in vitro the Process in Couteracted by Plasma Antithrombin and alpha-2-macroglobulin. J Fish Dis **15:**215-227.

230. **Sandkvist, M.** 2001. Biology of Type II Secretion. Mol Microbiol **40:**271-283.

231. **Sargent, F., Berks, B.C., Palmer, T.** 2006. Pathfinders and Trailblazers: a Prokaryotic Targeting System for Transport of Folded Proteins. FEMS Microbiology Letters **254:**198-207.

232. **Saulino, E. T., Bullitt, E., Hultgren, S.J.** 2000. Snapshots of Usher-mediated Protein Secretion and Ordered Pilus Assembly. Proceedings of the National Accademy of Sciences **97:**9240-9245.

233. **Schechter, L. M., Lee, C.A.** 2001. AraC/XylS Family Members, HilC and HilD, Directly Bind and Derepress the Salmonella typhimurium hilA promoter. Mol Microbiol **40:**1289-1299.

234. **Scherer, C. A., Cooper, E., Miller, S.I.** 2000. The Salmonella Type III Secretion Translocon Protein SspC is Inserted into the Epithelial Cell Plasma Membrane Upon Infection. Molecular Microbiology **37:**1133-1145.

235. **Schliwa, M., Euteneuer, U.** 1978. A microtubule-independent component may be involved in granule transport in pigment cells. Nature **273:**556-558.

236. **Schoehn, G., Di Guilmi, A.M., Lemaire, D., Attree, I., Weissenhorn, W., Dessen, A.** 2003. Oligomerisation of Type III Secretion Proteins PopB and PopD Precedes Pore Formation in Pseudomonas. EMBO **22:**4957-4967.

237. **Schubert, S., Rakin, A., Heesemann, J.** 2004. The Yersinia High-pathogenicity Island (HPI): Evolutionary and Functional Aspects. International Journal of Medical Microbiology **294:**83-94.

238. **Shea, J. E., Hensel, >m, Gleeson, C. and Holden, D.W.** 1996. Identification of a Virulence Locus Encoding a Second Type III Secretion System in *Salmonella typhimurium*. Proceedings of the National Accademy of Sciences **93:**2593-2597.

239. **Skerfving, S.** 1991. Exposure to mercury in the population, p. 411-425, Advances in mercury toxicology. Plenum Press, New York.

240. **Smart, G.** 1976. The Effect of Ammonia Exposure on Gill Structure of the Rainbow Trout (*Salmo gairdneri*). J. Fish Biol. **8:**471-475.

241. **Smith, A. H., Hopenhayn-Rich, C., Bates, M.N., Goeden, H.M., Hertz-Picciotto, I., Duggan, H.M., Wood, R., Kosnett, M.J., Smith, M.T.** 1992. Cancer risks from arsenic in drinking water. Environ Health Perspect **97:**259-267.

242. **Soto, G. E., Hultgren, S.J.** 1999. Bacterial Adhesins: Common Themes and Variations in Architecture and Assembly. J. Bacteriol. **181:**1059-1071.

243. **Spector, M. P.** 1998. The Starvation-stress Response (SSR) of Salmonella. Adv Microb Physiol **40:**233-279.

244. **Stathopoulos, C., Hendrixson, D.R., Thanassi, D.G., Hultgren, S.J., St Geme III, J.W., Curtiss III, R.** 2000. Secretion of Virulence Determinants by the General Secretory Pathway in Gram-negative Pathogens: an Evolving Story. Microbes Infect **2:**1061-1072.

245. **Stebbins, C. E., Galan, J.E.** 2000. Modulation of Host Signaling by a Bacterial Mimic: Structure of the Salmonella Effector SptP Bound to Rac1. Mol Cell **6:**1449-1460.

246. **Steele-Mortimer, O. e. a.** 2002. The Invasion-associated Type III Secretion System of Salmonella enterica serovar Typhimurium is Necessary for Intracellular Proliferation and Vacuole Biogenesis in Epithelial Cells. Cellular Microbiology **4:**43-54.

247. **Stender, S. e. a.** 2000. Idenfication of SopE2 from Salmonella typhimurium, a Conserved Guanine Nucleotide Exchange Factor for Cdc42 of the Host Cell. Molecular Microbiology **36:**1206-1221.

248. **Stevenson, R. M. W., Daly, J.G.** 1982. Biochemical and serological characteristics of Ontario isolates of *Yersinia ruckeri*. Can J Fish Aquat Sci **39:**870-876.

249. **Straus, D. B., W. A. Walter, and C. A. Gross.** 1987. The heat shock response of E. coli is regulated by changes in the concentration of [sigma]32. Nature **329:**348-351.

250. **Stuber, K., S. E. Burr, M. Braun, T. Wahli, and J. Frey.** 2003. Type III Secretion Genes in Aeromonas salmonicida subsp. salmonicida Are Located on a Large Thermolabile Virulence Plasmid. J. Clin. Microbiol. **41:**3854-3856.

251. **Sun, J., Hober, M.E., Rao, A.S., Neish, A.S., Madara, J.L.** 2004. Bacterial Activation of Beta-catenin Signaling in Human Epithelia. Am. J. Physiol. Gastrointest. Liver Physiol. **287:**G220-227.

252. **Suzuki, T., Imura, N., Clarkson, T.W.** 1991. Overview, p. 1-32. *In* T. Suzuki, Imura, N., Clarkson, T.W. (ed.), Advances in mercury toxicology. Plenum Press, New York.

253. **Sweet, L. I., and J. T. Zelikoff.** 2001. TOXICOLOGY AND IMMUNOTOXICOLOGY OF MERCURY: A COMPARATIVE REVIEW IN FISH AND HUMANS, p. 161-205, Journal of Toxicology & Environmental Health: Part B, vol. 4. Taylor & Francis Ltd.

254. **Thaler, C. D., Haimo, L.T.** 1992. Control of organelle transport in melanophores: regulation of Ca2+ and cAMP levels. Cell Motil. Cytoskeleton **22:**175-184.

255. **Tolleson, W. H.** 2005. Human melanocyte biology, toxicology and pathgology. J. Environ. Sci. Health C **23:**105-161.

256. **Toze, S.** 1999. PCR and the Detection of Microbial Pathogens in Water and Wastewater. Water Res **33:**3545-3556.

257. **Traci L. Testerman, Andrés Vazquez-Torres, Yisheng Xu, Jessica Jones-Carson, Stephen J. Libby, and Ferric C. Fang.** 2002. The alternative sigma factor σ<sup>E</sup> controls antioxidant defences required for Salmonella virulence and stationary-phase survival. Molecular Microbiology **43:**771-782.

258. **Trizio, D., Basketter, D.A., Botham, P.A., Graepel, P.H., Lambre, C., Magda, S.J., Pal, T.M., Riley, A.J., Ronneberger, H.** 1988. Identification of immunotoxic effects of chemicals and assessment of their relevance to man. Food Chem Toxicol **26:**527-539.

259. **Troisfontaines, P., and G. R. Cornelis.** 2005. Type III Secretion: More Systems Than You Think. Physiology **20:**326-339.

260. **Trust, T. J.** 1975. Bacteria associated with the gills of salmonid fishes in freshwater. J Appl Bacteriology **38:**225-233.

261. **Trust, T. J., E. E. Ishiguro, H. Chart, and W. W. Kay.** 1983. VIRULENCE PROPERTIES OF Aeromonas salmonicida. Journal of the World Mariculture Society **14:**191-200.

262. **Tseng, W.-P., Chu, H.M., How, S.W., Fong, J.M., Lin, C.S., Yeh, S.** 1968. Prevalence of skin cancer in an endemic area of chronic arsenicism in Taiwan. J Natl Cancer Inst **40:**453-463.

263. **Tsolis, R. M., Adams, L.G., Ficht, T.A., Baumier, A.J.** 1999. Contribution of Salmonella typhimurium Virulence Factors to Diarrheal Disease in Calves. Infect. Immun. **67:**4879-4885.

264. **van der Schalie, W. H., James, R.R., Gargan II T. P.** 2006. Selection of a Battery of Rapid Toxicity Sensors for Drinking Water Evaluation. Biosensors and Bioelectronics.

265. **van der Velden, A. W., Lindgren, S.W., Worley, M.J., Heffron, F.** 2000. Salmonella Pathogenicity Island 1-independent Induction of Apoptosis in Infected Macrophages by Salmonella enterica serotype Typhimurium. Infect. Immun. **68:**5702-5709.

266. **van der Velden, A. W., Velasquez, M., Starnbach, M.N.** 2003. Salmonella Rapidly Kill Dendritic Cells via a Caspase-1-dependent Mechanism. J Immunol **171:**6742-6749.

267. **Vazquez-Torres, A. e. a.** 1999. Extraintestinal Dissemination of Salmonella by CD18-expressing Phagocytes. Nature **401:**804-808.

268. **Vetsch, M., Puorger, C., Spirig, T., Grauschopf, U., Weber-Ban, E.U., Glockshuber, R.** 2004. Pilus Chaperones Represent a New Type of Protein-folding Catalyst. Nature **431:**329-333.

269. **Voetsch, A. C., Van Gilder, T.J., Angulo, F.J., Farley, M.M., Shallow, S., Marcus, R., Cieslak, P.R., Deneen, V.C., Tauxe, R.V.** 2004. FoodNet Estimate of the Burden of Illness Caused by Nontyphoidal Salmonella Infections in the United States. Clin Infect Dis **38:**S127-S134.

270. **von Frisch, K.** 1911. Beitrage zur Physiologie der Pigmentzellen in der Fischhaut. Pflug Arch Physiol **138:**319-387.

271. **Wall, D. M., Nadeau, W.J. Pazos, M.A., Shi, H.N., Galyov, E.E., McCormick, B.A.** 2007. Identification of the Salmonella enterica serotype Typhimurium SipA Domain Responsible for Inducing Neutrophil Recruitment Across the Intestinal Epithelium. Cell Microbiol **9:**2299-2313.

272. **Wallbrunn, H. M.** 1957. Genetics of the Siamese Fighting Fish, *Betta splendens*. Genetics **43:**289-298.

273. **Walsh, A. H., Ribelin, W.E.** 1975. The Pathology of Pesticide Poisoning, p. 515-557. *In* W. E. Ribelin, Migaki, G. (ed.), The Pathology of Fishes. The University of Wisconsin Press, Madison.

274. **Weston, C. R., Davis, R.J.** 2007. The JNK Signal Transduction Pathway. Curr Opin Cell Biol **19:**142-149.

275. **Winn, R. N.** 2001. Transgenic Fish as Models in Environmental Toxicology. Institute for Laboratory Animal Research Journal **42:**322-329.

276. **Winton, J. R., Rohovec, J.S., Fryer, J.L.** 1983. Bacterial and viral diseases of cultured salmonids in the Pacific Northwest.

277. **Wong, K. K., McClelland, M., Stillwell, L.C., Sisk, E.C., Thurston, S.J., Saffer, J.D.** 1998. Identification and Sequence Analysis of a 27-kilobase Chromosomal Fragment Containing a Salmonella Pathogenicity Island Located at 92min on the Chromosome Map of Salmonella enterica serovar typhimurium LT2. Infect. Immun. **66:**3365-3371.

278. **Wong, N. A., Pignatelli, M.** 2002. Beta-catenin, a Linchpin in Colorectal Carcinogenesis? Am J. Pathol. **160:**389-401.

279. **Wood, E. M., Yasutake, W.T.** 1956. Histopathology of kidney disease in fish. American Journal of Pathology **32:**845-857.

280. **Yeh, S., How, S.W., Lin, C.S.** 1968. Arsenical cancer of skin - histological study with special reference to Bowen's disease. Cancer **21:**312-339.

281. **Zahrl, D., Wagner, M., Bischof, K., Bayer, M., Zavecz, B., Beranek, A., Ruckenstuhl, C., Zarfel, G.E., Koraimann, G.** 2005. Peptidoglycan Degradation by Specialized Lytic Transglycosylases Associated with Type III and Type IV Secretion Systems. Microbiology **151:**3455-3467.

282. **Zeng, H., Wu, H., Sloane, V., Jones, R., Yu, Y., Lin, P. et al.** 2006. Flagellin/TLR5 Responses in Epithelia Reveal Intertwined Activation of Inflammatory and Apoptotic Pathways. Am J. Physiol Gastrointest Liver Physiol **290:**G96-G108.

283. **Zhang, X. L., Morris, C., Hackett, J.** 1997. Molecular Cloning, Nucleotide Sequence, and Function of a Site-specific Recombinase Encoded in the Major Pathogenicity Island of Salmonella typhi. Gene **202:**139-146.

284. **Zhou, D., Chen, L.M., Hernandez, L., Shears, S.B., Galan, J.E.** 2001. A Salmonella Inositol Polyphosphatase Acts in Conjunction with Other Bacterial Effectors to Promote Host Cell Actin Cytoskeleton Rearrangements and Bacterial Internalization. Molecular Microbiology **39:**248-259.

285. **Zhou, D., Mooseker, M.S., Galan, J.E.** 1999. Role of the S. typhimurium Actin-binding Protein SipA in Bacterial Internalization. Science **283:**2092-2095.

# APPENDIX

Biographical Information

Stephanie Renee Dukovcic was born on April 15, 1982 in Dublin, Ohio to Deborah A. Collucci and David T. Dukovcic. She spent most of her childhood in Dublin and relocated with her family to Oregon in 1997. Stephanie graduated from West Linn High School in 2000 and earned a B.S. in bioengineering with a minor in chemistry and a certificate in applied ethics from Oregon State University in 2005. In the fall of 2005, she began graduate training in microbiology at Oregon State University in the laboratory of Dr. Janine E. Trempy. She was the recipient of several scholarships [Supplemental Oregon Laurels (2008), Oregon Lottery (2008), Harriet M. Winton (2007) and the Middlekauf Outstanding Graduate Teaching and Service (2006) scholarships]. She also obtained a predoctoral fellowship from the Oregon Department of Fish and Wildlife to support her research efforts (2007-2009). Stephanie was also awarded the National Science Foundation Graduate Research Fellowship Honorable Mention in 2006. During her graduate training, she worked as a consultant for TriMike Creations during the summer of 2008. Stephanie is an avid soccer player and a volunteer ski patroller for the Santiam Pass Ski Patrol at Hoodoo, Oregon.

Publications

**Hutchison, J.R., Dukovcic, S.R., Dierksen, K.P., Carlyle, C.A., Caldwell, B.A. and Trempy, J.E.** 2008. Erythrophore cell response to food-associated pathogenic bacteria: implications for detection. Journal of Microbioal Biotechnology. 1:425-431.

**Dukovcic, S.R., Hutchison, J.R. and Trempy, J.E.** Characterization of a melanophore sensor derived from salmonids. In preparation 2009.

**Dukovcic, S.R., Hutchison, J.R. and Trempy, J.E.** Chinook melanophore response to water-based contaminants and bacterial pathogens. In preparation 2009.

**Dukovcic, S.R., Hutchison, J.R., Dierksen, K.P. and Trempy, J.E.** Erythrophore response to *Salmonella typhimurium*. In preparation 2009.

**Hutchison, J.R., Dukovcic, S.R., and Trempy, J.E.** Characterization of erythrophore cells to *Bacillus* group members. In preparation 2009.

Abstracts

Chinook melanophore cell-based biosensor. Dukovcic, S.R., Hutchison, J.R. and Trempy, J.E. 2009. Department of Microbiology Poster Session, Corvallis, OR.

Erythrophore cell response to Bacillus group members. Hutchison, J.R., Dierksen, K.P., Dukovcic, S.R. and Trempy, J.E. 2009. Department of Microbiology Poster Session, Corvallis, OR.

Erythrophore cell response to food-associated pathogenic bacteria: implications for detection. Hutchison, J.R., Dukovcic, S.R., Dierksen, K.P., Carlyle, C.A., Caldwell, B.A. and Trempy, J.E. 2009. Molecular and Cellular Biology Symposium, Corvallis, OR.

Characterization of erythrophore cell response to *Bacillus cereus*. Hutchison, J.R., Austin, M.J., Dukovcic, S.R., Carlyle, C.A., Caldwell, B.A. and Trempy, J.E. 2008. Oregon State University, Department of Microbiology Poster Session and Molecular and Cellular Biology Poster Session, Corvallis, OR. **Received award for best student poster Department of Microbiology.**

Immortalization of chromatophore cells. Dukovcic, S.R., Hutchison, J.R., Lowry, M. and Trempy, J.E. 2008. Department of Microbiology Poster Session. Corvallis, OR.

Characterization of a Chinook melanophore biosensor. Dukovcic, S.R., Hutchison, J.R., Caldwell, B.A. and Trempy, J.E. 2007. Department of Microbiology Poster Session. Corvallis, OR.

Characterization of the salmonid melanophore detection system. Hutchison, J.R., Dukovcic, S.R. and Trempy, J.E. 2007. 5th Annual Symposium: research advances in fisheries, wildlife and ecology. Corvallis, OR.

Biosensor response to pathogenic bacteria. Hutchison, J.R., Austin, M.J., Smith, R., Dukovcic, S.R., Caldwell, B.A. and Trempy, J.E. 2006. Center for Genome Research and Biocomputing Annual Retreat. Redmond, OR. **Received award for best student poster.**

Biosensor response to pathogenic bacteria. Hutchison, J.R., Austin, M.J., Smith, R., Dukovcic, S.R., Caldwell, B.A and Trempy, J.E. 2006. American Society of Microbiology National Meeting. Orlando, FL.