Five Year Strategic Research Work Plan 2014 – 2019

U. S. Fish and Wildlife Service
Aquatic Animal Drug Approval Partnership Program

Program Overview:

The U. S. Fish and Wildlife Service (Service) Aquatic Animal Drug Approval Partnership Program (AADAPP) was established in 1994 to solve a crisis created when the U. S. Food and Drug Administration (FDA) began to enforce the Federal Food, Drug, and Cosmetic Act and FDA regulations regarding the use of drugs in Minor Species, including fish. These FDA decisions effectively stripped the National Fish Hatchery System (NFHS) of virtually all the tools that it had used for decades to manage fish health and achieve Service fish culture, fisheries management, and fisheries research objectives. AADAPP's goal was to work within the newly enforced regulatory framework and put these essential tools back in the hands of Service personnel who needed them.

The AADAPP Research Program (RP) generates high quality data to support the approval of fish drugs by the U. S. Food and Drug Administration (FDA). Guiding safe and effective fish drugs "through the approval pipeline" and into the hands of Service and other fisheries professionals is of the utmost importance because it makes accessing and using fish drugs simpler and also demonstrates the Service's good faith and commitment to the drug approval effort. Obtaining FDA approval for use of a drug on food animals, including fish, is a long, arduous, and expensive process (e.g., $10-20M and 15-30 years). The FDA approval process that AADAPP follows has become the "gold standard" throughout the world because of its rigorous approach to protecting the public and preventing unsafe or ineffective drugs from reaching the marketplace. The AADAPP RP has taken lead responsibility to establish safe and effective treatment regimens for many drugs that have been approved by FDA. Over time, AADAPP researchers have developed expertise in regulatory science and the ability to conduct research in compliance with Good Clinical Practices and Good Laboratory Practice (GLP) requirements.

The current Strategic Plan addresses the AADAPP Mission, and is focused on demonstrating the effectiveness and target animal safety of various fish drugs needed by fisheries professionals. Specifically, the objectives described herein address ongoing and emerging needs for antimicrobials, antibiotics, and sedatives.

Research Unit
AADAP Research Team

Location
Bozeman Fish Technology Center
Bozeman, Montana

Investigators and FTEs

James D. Bowker	Research Program Manager, Fishery Biologist	1.0
Daniel Carty	Fishery Biologist	0.5
Molly P. Bowman	Fishery Biologist	1.0
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Project Summary:

There are currently an insufficient number of fish drugs approved by FDA and available to allow fisheries professionals and researchers to meet fish culture, fish health, fishery management, and fishery research goals. Obtaining new approvals for drugs such as antimicrobials, antibiotics, sedatives, spawning aids, and marking agents is crucial to ensure that the proper tools are available to deal with issues related to aquaculture, propagation, and fish handling. The goal and objectives described below directly support FDA approval of new fish drugs or expansion of new claims for fish drugs already approved. Trials will be conducted with a number of fish drugs to evaluate their safety and effectiveness on a variety of representative fish species.

The anticipated outcomes from the project are:

1. Demonstration of the effectiveness of an oral antibiotic to control mortality caused by specific bacterial fish pathogens
2. Demonstration of the effectiveness of a water-borne antibiotic to control mortality caused by columnaris;
3. Demonstration of the effectiveness of a water-borne antimicrobial to control mortality caused by external columnaris;
4. Demonstration the effectiveness of a sedative to sedate seawater fish to handleable;
5. Demonstration of the effectiveness of a sedative to lightly sedate both freshwater and seawater fish.

Results from studies will be summarized in Final Study Reports that will be submitted to FDA for review and concurrence. Letters from FDA stating concurrence will be provided to the drug sponsor to facilitate their efforts to obtain approval of a new animal drug or a new claim for a currently approved drug.

Goal and Objectives

The goal of this project is to generate data to support FDA-approval of drugs critically needed by fish culturists, fish health biologists, fishery researchers, and fisheries managers. This will be accomplished through collaborative research with cooperators where field studies can be conducted. A major difficulty in generating effectiveness data is identifying locations (e.g., labs, hatcheries, academic institutions) where studies can be conducted. In the case of evaluating the effectiveness of therapeutants, locations need to be identified that have recurring, somewhat predictable disease outbreak involving the desired test fish species, adequate water flow to conduct the study, and an adequate number of test tanks to conduct small-scale studies.

Objectives are:

Objective 1: Identify locations/cooperators and conduct trials to demonstrate the effectiveness of Aquaflor (50% florfenicol) to control mortality in freshwater salmonids due to bacterial kidney disease.

Objective 2: Identify locations/cooperators and conduct trials to demonstrate the effectiveness of Aquaflor (50% florfenicol) to control mortality in seawater salmonids due to bacterial kidney disease, furunculosis, or yellowmouth disease (or tenacibaculosis).

Objective 3: Identify locations/cooperators and conduct trials to demonstrate the effectiveness of Pennox 343 (oxytetracycline-hydrochloride) to control mortality in freshwater nonsalmonids due to columnaris.

Objective 4: Identify locations/cooperators and conduct trials to demonstrate the effectiveness of Halamid (chloramine-T) to control mortality in coolwater fish due to columnaris.

Objective 5: Identify locations/cooperators and conduct trials to demonstrate the effectiveness of Aqui-S20E (10% eugenol) to lightly sedate or sedate seawater finfish to handleable.

Objective 6: Identify locations/cooperators and conduct trials to demonstrate the effectiveness of Aqui-S20E (10% eugenol) to lightly sedate freshwater salmonids.

Objective 7: Conduct trials in accordance with Good Laboratory Practice regulations to demonstrate the safety of Aqui-S20E (10% eugenol) to lightly sedate freshwater salmonids.

Objective 8: Conduct trials in accordance with Good Laboratory Practice regulations to demonstrate the safety of Aqui-S20E (10% eugenol) to lightly sedate or sedate seawater finfish to handleable.

Need for Research

There are only eight drugs currently approved for use on fish that can enter the human food chain through release into public waters (i.e., the wild) or commercial production. In the case of therapeutants, approvals are restricted to certain fish diseases (e.g., coldwater disease) in certain classes of fish (e.g., salmonids). Use of the only FDA- approved fish sedative (tricaine methanesulfonate) is restricted by the fact that fish must be withheld for 21 d before they can be released into public waters. As such, fisheries professionals do not have an adequately stocked medicine chest filled with a sufficient number of drugs that can be used for a variety of purposes (e.g., to control mortality caused by coldwater disease, columnaris, bacterial kidney disease, and yellowmouth disease). The paucity of FDA-approved drugs jeopardizes Service fisheries projects and has the potential to negatively affect the ability of managers to achieve Service goals. If the AADAPP research team is able to identify study locations and secure commitments from study collaborators to conduct studies to address each of the proposed objectives, new approvals could be obtained or substantial progress toward approval could be made. Additional approvals for Aquaflor are needed because there are no antibiotics currently approved for the claims listed above. Broad approvals are critically needed for Pennox 343 and Halamid to allow treatment of salmonids and nonsalmonids to control mortality caused by columnaris. Last, a new approval is desperately needed for an immediate-release fish sedative where fish can be returned to public waters immediately after they recover from sedation. Legal and judicious use of therapeutants when they become approved will likely result in an increase in the number of fish that survive a disease outbreak and will improve overall fish health quality of fish being stocked in public waters. Use of the fish sedative when it is approved will allow fisheries professionals, perhaps for the first time, to be able to sedate fish for a variety of purposes and immediately release treated fish into public waters, thereby conducting research in accordance with the Federal Food, Drug, and Cosmetic Act.

Scientific Background

Objective 1: Demonstrate the effectiveness of Aquaflor to control mortality in freshwater salmonids due to bacterial kidney disease. Bacterial kidney disease (BKD), caused by *Renibacterium salmoninarum* (Sanders and Fryer 1980), is a serious disease of cultured (Earp et al. 1953) and feral salmonids (Smith 1964) and is widespread throughout North America, Chile, Europe, and Japan (Pascho and Elliott 2003). The disease (1) is a systemic infection that is normally slowly progressive and frequently fatal, and (2) seldom shows up in fish until they are 6 – 12 months old (Inglis et al. 1993). Fish severely affected with BKD may or may not show external clinical signs (e.g., pale gills, exophthalmia, abdominal extension, skin blisters, shallow ulcers, or hemorrhages). Internally, infected fish are most frequently observed with creamy-white granulomatous lesions in the kidney (Inglis et al. 1993).

Until recently, erythromycin has been identified as the antibiotic of choice for treating BKD, and a 28-d oral regimen was recommended for treating young hatchery fish (Wolf and Dunbar 1959). Although not yet FDA-approved, Aquamycin 100 (erythryomycin thiocyanate, Bimeda, A Division of Cross Vetpharm Group, Ltd., 2836 Dolliver Park Avenue, Lehigh, IA 50557) is the only product currently used for treatment of BKD. However, the FDA-approval process for this drug is taking longer than anticipated, treatment efficacy has been somewhat inconsistent, and some investigators have noted signs of toxicity (e.g., tetany and jaundice) in treated fish. Consequently, fisheries professionals are evaluating the effectiveness of alternative antibiotics, preferably those that are already approved by FDA for use in salmonids.

Florfenicol is an antibiotic that is a potential alternative to erythromycin. Florfenicol is (1) a potent, broad-spectrum, antimicrobial agent with bacteriostatic and bacteriocidal properties (Horsberg et al. 1996), (2) a fluorinated analogue of thiamphenicol and similar in structure to chloramphenicol, both of which have been used as broad-spectrum, veterinary antibiotics (Nagata and Oka 1996), (3) has great potential for treatment of infectious diseases, and—because of its high potency, safety to humans, and the fact that it is not used in human medicine—its importance in veterinary medicine will likely increase, especially with respect to animals used by humans for food (Powers et al. 1990), and (4) the FDA recently approved Aquaflor for use as an antimicrobial for the following indications: for control of mortality in (a) catfish due to enteric septicemia associated with *Edwardsiella ictaluri,* (b) warmwater finfish due to streptococcal disease associated with *Streptococcus iniae* (Bowker et al. 2010), and (c) for control of mortality in freshwater-reared salmonids due to (i) coldwater disease associated with *Flavobacterium psychrophilum*, (ii) furunculosis disease associated with *Aeromonas salmonicida*, and (iii) columnaris disease associated with *F. columnare*. These approvals have generated considerable interest in expanding the Aquaflor label to include additional antimicrobial uses in aquaculture, e.g., to allow its use to control mortality in freshwater-reared salmonids due to BKD associated with *R. salmoninarum*.

Two studies have already been conducted to evaluate the effectiveness of Aquaflor administered at a dosage of 15 mg florfenicol/kg fish body weight for 10 d to control mortality in Spring Chinook salmon caused by BKD. Although FDA concurred with both study's conclusions that treatment was effective, they require one more study in which a different study investigator is responsible for conducting the study. Therefore, if funding is available, we will conduct one additional study to demonstrate the effectiveness of Aquaflor to control mortality in Spring Chinook salmon.

Objective 2: Demonstrate the effectiveness of Aquaflor to control mortality in seawater salmonids due to bacterial kidney disease, furunculosis, and yellowmouth disease. See Scientific Background Objective 1 for scientific background on BKD and please note that although BKD was initially recorded in freshwater situations, it is now recognized as a costly

problem in the seawater farming of salmonids, particularly in the Pacific Northwest (Inglis et al. 1993).

Furunculosis is one of the oldest known bacterial fish diseases and is generally considered a disease of salmonids (Plumb 1999). Mortality in affected freshwater-reared salmonid populations and resultant economic losses to producers can be substantial (Clark and Scott 1989). Hence, maintaining healthy rearing conditions, administering preventative vaccines, and administering antimicrobial treatments are strategies routinely used to prevent furunculosis outbreaks or minimize mortality when outbreaks occur (Inglis et al. 1991). Aqauflor is currently approved by the FDA for use on freshwater salmonids. However, no antibiotic is approved in the United States to control mortality of seawater salmonids due to columnaris disease.

Yellowmouth disease (also called tenacibaculosis) associated with*Tenacibaculum maritimum*, a Gram-negative and filamentous bacterium, has been described as the etiological agent of tenacibaculosis in marine fish. The pathology of the disease caused by this marine organism has mainly been associated with characteristic gross lesions on the body surface of fish such as ulcers, necrosis, eroded mouth, frayed fins and tail rots, and sometimes necrosis on the gills and eyes (McVicar and White 1979; Campbell and Buswell 1982; Baxa et al. 1986; Devesa et al. 1989; Alsina and Blanch 1993; Chen et al. 1995; Handlinger et al. 1997; Ostland et al. 1999; Cepeda and Santos 2002). Tenacibaculosis is one of the most threatening bacterial infections limiting the culture of many species of commercial value in distinct geographical areas of the world (see review by Toranzo et al. 2005). Marine tenacibaculosis infection was first described by Masumura and Wakabayashi (1977) as the cause of mortalities in red Pagrus major and black sea bream *Acanthopagrus schlegeli*, when a massive epizootic occurred in a hatchery in Hiroshima Prefecture (Japan). A few years later, the disease spread to other important cultured fish species in Japan such as Japanese flounder *Paralichthys olivaceous* and yellowtail *Seriola quinqueradiata*, among others (Baxa et al. 1986; Wakabayashi et al. 1986). Since then, outbreaks of tenacibaculosis have been reported in other geographical areas of the world, including North America, affecting Atlantic salmon smolts from British Columbia, Canada (Ostland et al. 1999). Recently, tenacibaculosis has been blamed for increased mortality of Atlantic salmon in the Pacific Northwest. No antibiotics are approved in the United States for this disease.

Therefore, if funding is available, we will identify study locations/cooperators and conduct multiple studies to demonstrate that Aquaflor is effective in controlling mortality in (a) a variety of seawater reared salmonids caused by furunculosis, (b) Chinook salmon caused by BKD, and (c) Atlantic salmon caused by tenacibaculosis.

Objective 3: Identify locations/cooperators and conduct trials to demonstrate the effectiveness of Pennox 343 (oxytetracyline-hydrochloride) to control mortality in freshwater nonsalmonids due to columnaris. Columnaris (causative agent, *Flavobacterium columnare*) is an acute-to-chronic external or systemic bacterial disease affecting freshwater-reared finfish worldwide (Bullock et al. 1986). Infections begin when *F. columnare* invades epithelial tissues, most commonly affecting the gills and buccal, opercular, dorsal, and caudal surfaces (Post 1987). Lesions form as the infection progresses and are often observed at the base of the dorsal fin or on the caudal fin, lending columnaris its common names, “saddleback” and “fin rot.” If left untreated, external lesions may penetrate blood vessels or the body cavity, leading to systemic infections.

Columnaris can be presumptively diagnosed based on clinical signs, including the presence of the aforementioned lesions and long, slender, possibly filamentous, rod-shaped, Gram-negative

bacteria (Post 1987) exhibiting the characteristic “haystack” formation and “flexing” behavior of *F. columnare* (Noga 2000). In virtually all instances, columnaris outbreaks require intervention to prevent significant losses. Shedding of bacteria from the epithelial surfaces of infected fish may create a self-perpetuating, population-wide infection (Post 1987). If left untreated, mortalities in overcrowded or unsanitary conditions may reach 70% or higher among young and most-susceptible fishes (Post 1987). During outbreaks, fish culturists can often minimize mortality by improving environmental rearing conditions, administering chemotherapeutic bath treatments, or both.

Oxytetracycline is a broad-spectrum antibiotic produced by the actinomycete *Streptomyces rimosus.* It is a bacteriostatic compound widely used in veterinary medicine, partly due to its lower order of toxicity and ability to readily distribute into blood and most tissues (Barragry 1994). As a member of the tetracycline family, it is used mainly in treating infections caused by Gram-negative bacterial pathogens (Rigos et al. 2006). Since its isolation and development in 1950, OTC has become one of the most commonly used antibiotics in aquaculture (Xu and Rogers 1994; Rigos et al. 2006). OTC is effective against many fish pathogens in many fish species, including (a) flavobacterial infections, furunculosis (Cipriano et al. 1996), ulcer disease, and enteric redmouth disease (Plumb 1999) in salmonids, (b) bacterial hemorrhagic septicemia and pseudomonas disease in salmonids and catfish, (c) vibriosis in rainbow trout (Post 1987; Hughes et al. 1990; Plumb 1999), (d) *Streptococcus iniae* in blue tilapia *Tilapia aurea* (Darwish et al. 2002), and (e) *Edwardsiella ictaluri* in catfish (Inglis et al. 1993). However, for most of the aforementioned fish species, OTC treatment is typically delivered via medicated feed or injection. The administration of medicated feed can be problematic when (1) fish appetite decreases during incidences of disease, (2) hierarchical fish behavior in intensive culture systems prevents even distribution of the therapeutic dose, or (3) early life stage fish are yet accustomed to feeding. Drug administration via injection can be labor intensive, costly, and oftentimes impractical when treatment of the whole population is necessary. Conversely, bath treatments are relatively easy to administer and do not require handling fish. These treatments can be used to treat young and inappetent fish; when administered properly, bath treatments deliver a more uniform dose. Currently, Pennox 343 is not approved by the FDA for use on fish as a therapeutants.

Therefore, if funding is available, we will identify study locations/cooperators and conduct multiple studies to demonstrate that Pennox 343 is effective in controlling mortality in a variety of nonsalmonids caused by columnaris.

Objective 4: Identify locations/cooperators and conduct trials to demonstrate the effectiveness of Halamid (chloramine-T) to control mortality in coolwater fish due to columnaris. See Objective 4 for information on columnaris disease. A number of external sanitizing agents, including chloramine-T (CLT) have been used to control mortality caused by *Flavobacterium* spp. in a variety of freshwater finfish, including *F. branchiophila* (a causative agent of bacterial gill disease; BGD) and *F. columnare*. Chloramine-T (C7H7ClNNaO2S•3H2O) is a biocide used worldwide as a disinfectant and antiseptic. Although CLT has been used for years under authorization of publically held Investigational New Animal Drug (INAD) exemptions and has been shown to effectively control mortality associated with BGD in freshwater-reared salmonids (From 1980; Speare and Ferguson 1989; Bullock et al. 1991; Thorburn and Moccia 1993; Ostland et al. 1995; Bowker and Erdahl 1998; Bowker et al. 2008) and columnaris in a variety of freshwater finfish (Bowker et al. 2013), it is not yet approved by the U.S. Food and Drug Administration (FDA) for use on fish. At this time, sufficient efficacy data have been generated to support an initial Halamid approval to allow treatment to control mortality of warmwater fish and walleye caused by columnaris disease.

Therefore, if funding is available, we will identify study locations/cooperators and conduct multiple studies to demonstrate that Halamid is effective in controlling mortality in coolwater fish other than walleye caused by columnaris.

Objective 5: Identify locations/cooperators and conduct trials to demonstrate the effectiveness of Aqui-S20E (10% eugenol) to lightly sedate or sedate seawater finfish to handleable. Anesthetics or sedatives are chemicals or physical agents that—with increasing treatment concentration and duration—first calm an animal and then cause successive loss of mobility, equilibrium, consciousness, and reflex action. Availability of safe and effective fish sedatives is crucial to fisheries researchers, managers, and culturists. Fisheries professionals routinely sedate fish for procedures such as collection of samples or morphometric data, surgical implantation of tags or tracking devices, and transport. Fish are innately difficult to handle, and when a fish is actively resisting restraint, epithelial damage or other physical injury is more likely. Fish that are handled without sedation may also be negatively affected by the physiological consequences of stress. Fisheries professionals must also consider the issues of animal welfare, and that sedation is recommended for procedures that may cause undue stress.

Ideally, a fish sedative is safe, effective, easy to administer, has rapid induction and recovery times, offers some analgesia, can be used over a broad range of water chemistries, and is inexpensive. Additionally, it is often desirable that the sedative have no withdrawal period so that treated fish can be released into the wild or taken to market immediately after treatment. Currently, there are few sedative options available to fisheries professionals that are safe, effective, and practical to use, and there is no sedative that can be legally used as an immediate-release sedative. There is only one compound (tricaine methanesulfonate or "tricaine") approved by the U.S. Food and Drug Administration (FDA) for the temporary immobilization of fish and other aquatic, cold-blooded animals. Tricaine is generally considered to be safe and effective and is widely used by fisheries professionals for a variety of purposes. However, legal use is limited to four families of fish (Ictaluridae, Salmonidae, Esocidae, and Percidae) and water temperatures above 10°C, and a 21-d withdrawal period is required for fishes intended for human consumption or that could be caught and consumed by sport or commercial fishers. For many applications, holding fish for a lengthy period of time postsedation is not practical or seriously compromises management or research activities. Holding fish is especially problematic in field settings. To avoid these complications, an FDA-approved immediate-release sedative is desperately needed.

There is one compound currently being investigated for use as an immediate-release fish sedative - eugenol, which elicits sedative effects by interfering with changes in membrane permeability necessary to conduction of nervous stimuli. AADAP researchers and others (Trushenski et al. 2012a, 2012b, 2012c) have conducted safety and effectiveness studies with Aqui-S20E (10% eugenol) and are working with the sponsor to generate data to support approval of this product for use as an immediate-release sedative. Sufficient data have been generated on freshwater fish to complete the effectiveness technical section for this product to sedate freshwater fish to handleable and studies have been completed to demonstrate that there is an adequate margin of safety associated with sedating a variety of freshwater fish to handleable. To date, no such data have been generated to support approval of Aqui-S20E for use on seawater fish for any purpose.

Therefore, if funding is available, we will identify study locations/cooperators and conduct multiple studies to demonstrate that Aqui-S20E is effective to lightly sedate seawater fish for extended periods of time (e.g., 4 h) or sedate seawater finfish to handleable.

Objective 6: Identify locations/cooperators and conduct trials to demonstrate the effectiveness of Aqui-S20E (10% eugenol) to lightly sedate freshwater salmonids. See Objective 5 for information on the need for an immediate-release fish sedative. AADAP researchers have completed data requirements to demonstrate that Aqui-S20E safely and effectively sedates freshwater fish to handleable. To date, no such data has been generated to demonstrate that Aqui-S20E can safely and effectively lightly sedate freshwater fish for extended periods of time for purposes such as transport.

Therefore, if funding is available, we will identify study locations/cooperators and conduct multiple studies to demonstrate that Aqui-S20e is effective to lightly sedate freshwater fish.

Objective 7: Conduct trials in accordance with Good Laboratory Practice regulations to demonstrate the safety of Aqui-S20E (10% eugenol) to lightly sedate freshwater salmonids. AADAP is one of very few fisheries programs in the United States that is capable of conducting target animal safety (TAS) studies that are compliant with Good Laboratory Practice (GLP) regulations. The FDA requires that TAS studies conducted to support of new animal drug approvals be compliant with GLPs. Such studies will be required to support an approval to use Aqui-20E to lightly sedate fish for extended periods of time.

Therefore, if funding is available, we will conduct multiple studies to demonstrate that there is an adequate margin of safety associated with using Aqui-S20e to lightly sedate freshwater salmonids for extended periods of time.

Objective 8: Conduct trials in accordance with Good Laboratory Practice regulations to demonstrate the safety of Aqui-S20E (10% eugenol) to lightly sedate or sedate seawater finfish. AADAP is one of very few fisheries programs in the United States capable of conducting target animal safety (TAS) studies that are compliant with Good Laboratory Practice (GLP) regulations. The FDA requires that TAS studies conducted to support of new animal drug approvals be compliant with GLPs. Such studies will be required to support an approval to use Aqui-S20E to lightly sedate seawater fish for extended periods of time or to sedate seawater fish to handleable.

Therefore, if funding is available, we will conduct multiple studies to demonstrate that there is an adequate margin of safety associated with using Aqui-S20E to lightly sedate seawater fish for extended periods of time or to sedate seawater fish to handleable.

Related Research: This project is closely aligned with projects undertaken by AADAP research staff over the past 10-15 years. The expertise developed by AADAP staff during this period has resulted in (a) a high rate of success relative to submitting final study reports that are accepted by FDA, (b) completion of effectiveness and target animal safety technical sections, which have (c) ultimately been used by the drug sponsor to support new drug approvals or additional approvals for drugs already approved for use on fish. AADAP researchers are recognized as experts in developing detailed research study protocols, designing experiments to address regulatory requirements, collecting data in compliance with quality control standards, preparing final study reports with sufficient detail that FDA reviewers can confidently confirm procedures and verify results, and provide written communication to FDA in a manner consistent with their expectations. Based on AADAP's success in completing field effectiveness and target animal safety studies, and "passing" inspections by FDA Field Inspectors of GLP previously conducted studies, AADAP is frequently sought out to conduct studies to support fish drug approvals.

APPROACHES AND RESEARCH PROCEDURES

Objective 1: ***Demonstrate the effectiveness of Aquaflor to control mortality in freshwater salmonids due to bacterial kidney disease.*** (Lead: Bowman; Support: Bowker, Wandelear, and Carty).

The goal of this objective is provide confirmatory demonstration that Aquaflor is effective in controlling mortality of fingerling Chinook salmon due to BKD in a field experiment. Results that demonstrate effectiveness will definitively show that Aquaflor is effective for this claim. Studies will be conducted under study protocol AQFLR-09-EFF, *The Efficacy of AQUAFLOR® (50% Florfenicol; Type A Medicated Article) Administered in Feed to Control Mortality in Freshwater-Reared Finfish*. Note that two studies have been previously conducted under this protocol, and both demonstrated that Aquaflor administered at the prescribed dosage was effective in control mortality in Chinook salmon caused by BKD. However, the lead field investigator in both studies was the same person, and FDA recommends that the lead investigator in each study not be the same person. Therefore, one additional study is required in which the Investigator is not the same person as that in the first two studies.

Null Hypothesis. Mean percent total mortality in tanks (experimental units) of test fish treated with AQUAFLOR®-medicated feed at 15 mg florfenicol/kg fish/d for 10 d is equal ($P \geq 0.05$) to that in nontreated (control) test tanks.

Experimental Design. The study will include two treatment conditions (treated vs. nontreated control). Each treatment condition will be randomly assigned to at least 3 replicate test tanks (note that there will be an equal number of treated and nontreated control tanks); thus, at least 6 test tanks will be used in each study. Each test tank will contain ≥100 test fish (not that each tank will contain an equal number of fish). The study will consist of the following three periods:

(1) Pretreatment period (study days -*X* – 0; total days to be documented),
(2) Treatment period (study days 1 – 10; total days = 10), and
(3) Posttreatment period (study days 11 – 24; total days = 14)

Aquaflor medicated feed will be administered to fish in treated tanks at a dose of 15 mg florfenicol/kg fish/d on 10 consecutive days. The study will employ single masking. Data collection will include measuring water temperature and dissolved oxygen concentration daily, counting and removing dead fish daily, performing comprehensive fish health evaluations during the pretreatment, treatment, and posttreatment periods, collecting feed samples and having them analyzed to confirm that florfenicol was incorporated onto feed at the proper dose, and measure other water quality parameters periodically throughout the study. Mortality data will be analyzed by SAS Proc Glimmix.

Collaborations: Doug Munson and staff of the Idaho Department of Fish and Game will collaborate by providing test animals, and with experimental setup, study conduct and data collection, fish health evaluations, and collecting feed samples for dose verification.

Objective 2: Demonstrate the effectiveness of Aquaflor to control mortality in seawater salmonids due to bacterial kidney disease, furunculosis, and yellowmouth disease.
Lead: Bowker; Support: Bowman, Wandelear, Carty)

The goal of this objective is provide evidence that Aquaflor is effective in controlling mortality in seawater salmon due to BKD, furunculosis, and yellowmouth disease in field experiments. As per FDA requirements, at least two studies will need to be completed to demonstrate effectiveness for each claim. Studies will be conducted under study protocol AQFLR-14-SEA-EFF, *The Efficacy of AQUAFLOR® (50% Florfenicol; Type A Medicated Article) Administered in Feed to Control Mortality in Seawater-Reared Finfish*.

Null Hypothesis. Mean percent total mortality in tanks (experimental units) of test fish treated with AQUAFLOR®-medicated feed at 10 or 15 mg florfenicol/kg fish/d for 10 d is equal ($P \geq 0.05$) to that in nontreated (control) test tanks.

Experimental Design. Each study will be conducted in a manner nearly identical to that described for Objective 1, with the following exception. It is likely that studies conducted under this Objective will be conducted at net pen locations and test tanks will not be standard fiberglass or aluminum tanks used in studies on land.

Collaborations: Amir Rameriz and staff at American Gold will collaborate with trials to evaluate the efficacy to control mortality of Atlantic salmon caused by yellowmouth disease by providing test animals, and with experimental setup, study conduct and data collection, fish health evaluations, and collecting feed samples for dose verification. No other collaborators have been identified to help us conduct trials to evaluate the efficacy of Aquaflor to control mortality in seawater salmonids due to furunculosis or BKD. When identified, these individuals will collaborate by providing test animals, and with experimental setup, study conduct and data collection, fish health evaluations, and collecting feed samples for dose verification.

Objective 3: Identify locations/cooperators and conduct trials to demonstrate the effectiveness of Pennox 343 (oxytetracyline-hydrochloride) to control mortality in freshwater nonsalmonids due to columnaris. (Lead: Wandelear, Mike Matthews [Florida Bass Conservation Center Richloam Hatchery], and Coja Yamashita [Pennsylvania Fish and Boat Commission Benner Springs Hatchery); Support: Bowman, Bowker, Carty).

The goal of this objective is provide evidence that Pennox 343 is effective in controlling mortality in freshwater nonsalmonids due to columnaris in field experiments. As per FDA requirements, at least 3-4 studies will need to be completed to demonstrate effectiveness for nonsalmonids. Studies will be conducted under study protocol OTC-12-EFF-IMM, The Efficacy of Pennox 343 (Oxytetracycline hydrochloride) to Control Mortality Caused by Susceptible Pathogens of Freshwater-Reared Finfish.

Null Hypothesis. Mean percent total mortality in tanks (experimental units) of test fish treated with Pennox 343 at 20 mg/L OTC-HCL for 60 min daily on 3 consecutive d is equal ($P \geq 0.05$) to that in nontreated (control) test tanks.

Experimental Design. A study will include two treatment conditions (treated vs. nontreated control). Each treatment condition will be randomly assigned to at least 3 replicate test tanks; thus, at least 6 test tanks will be used in each study. A study will comprise three periods:
(a) Pretreatment (study days $-X$ – 0; total days to be reported in FSR),
(b) Treatment (study days 3), and
(c) Posttreatment (study days 4 – 17; total posttreatment days = 14).

Pennox 343 will be administered to fish in treated tanks at a dose of 40-50 mg/L OTC-HCL for 60 min daily on 3 consecutive days. The study will employ single masking. Data collection will

include measuring water temperature and dissolved oxygen concentration daily, counting and removing dead fish daily, performing comprehensive fish health evaluations during the pretreatment, treatment, and posttreatment periods, collecting water samples and having them analyzed to confirm that OTC was administered in water at the proper dose, and measure other water quality parameters periodically throughout the study. Mortality data will be analyzed by SAS Proc Glimmix.

Collaborations: Mike Matthews and staff at the Florida Bass Conservation Center Richloam Hatchery, and Coja Yamashita and staff at Pennsylvania Fish and Boat Commission Benner Springs Hatchery will collaborate by providing test animals, and with experimental setup, study conduct and data collection, fish health evaluations, and collecting water samples for dose verification.

Objective 4: Identify locations/cooperators and conduct trials to demonstrate the effectiveness of Halamid (chloramine-T) to control mortality in coolwater fish due to columnaris. (Lead: Wandelear, and Coja Yamashita [Pennsylvania Fish and Boat Commission Benner Springs Hatchery); Support: Bowman, Bowker, Carty, and others at the study site).

The goal of this objective is provide evidence that chloramine-T is effective in controlling mortality in freshwater nonsalmonids due to columnaris in field experiments. As per FDA requirements, at least 1 study will need to be completed to demonstrate effectiveness for nonsalmonids (three studies have already been conducted by AADAP and accepted by FDA with bluegill, largemouth bass, and walleye). Studies will be conducted under study protocol CHLT-07-EFF.1, "The efficacy of chloramine-T to control mortality due to bacterial gill disease or external columnaris in cool and warmwater finfish."

Null Hypothesis. Mean percent total mortality in tanks (experimental units) of test fish treated with Halamid at 20 mg/L chloramine-T for 60 min daily on 3 consecutive d is equal ($P \geq 0.05$) to that in nontreated (control) test tanks.

Experimental Design. A study will include two treatment conditions (treated vs. nontreated control). Each treatment condition will be randomly assigned to at least 3 replicate test tanks; thus, at least 6 test tanks will be used in each study. A study will comprise three periods:
(a) Pretreatment (study days -X – 0; total days to be reported in FSR),
(b) Treatment (study days 3), and
(c) Posttreatment (study days 4 – 17; total posttreatment days = 14).
Halamid will be administered to fish in treated tanks at a dose of 20 mg/L chloramine-T for 60 min daily on 3 consecutive days. The study will employ single masking. Data collection will include measuring water temperature and dissolved oxygen concentration daily, counting and removing dead fish daily, performing comprehensive fish health evaluations during the pretreatment, treatment, and posttreatment periods, collecting water samples and having them analyzed to confirm that chloramine-T was administered in water at the proper dose, and measure other water quality parameters periodically throughout the study. Mortality data will be analyzed by SAS Proc Glimmix.

Collaborations: Coja Yamashita and staff at Pennsylvania Fish and Boat Commission Benner Springs Hatchery will collaborate by providing test animals, and with experimental setup, study conduct and data collection, fish health evaluations, and analyzing water sam samples for dose verification.

Objective 5: Identify locations/cooperators and conduct trials to demonstrate the effectiveness of Aqui-S20E (10% eugenol) to lightly sedate or sedate seawater finfish to handleable. (Lead: Bowker, study site Investigator; Support: Bowman, Wandelear, Carty, and study site personnel).

The goal of this objective is provide evidence that Aqui-S20E is effective for lightly sedating seawater finfish for extended periods of time (e.g., up to 4 h) or for sedating to handleable. As per FDA requirements, studies on at least 6 different seawater fish species will likely need to be completed to demonstrate effectiveness for each claim. Studies to evaluate the effectiveness of Aqui-S20E to sedate fish to handleable will be conducted under study protocol AQS20E-14-SEA-EFF, *The Efficacy of AQUI-S®20E to Sedate a Variety of Finfish to Handleable in Seawater.* The research protocol to describe procedures for conducting studies to evaluate the effectiveness of Aqui-S20E to lightly sedate fish is in development.

Research Hypothesis. Sedation to handleable: At the AQUI-S®20E concentration tested, the percentage of fish that become sedated to handleable within 5 min is 80%. Lightly sedated: TBD.

Experimental Design. Sedation to handleable: To ensure masking, and to address the null hypothesis without bias, each of the groups of 30 fish will be broken-up into two rounds of 15 fish/round (a total of 4 rounds of 15 fish/round will be randomized). A completely randomized design will be used to determine the order in which the four rounds (2 rounds of AQUI-S®20E and 2 rounds of active control) will be administered.

During each round tested, 15 fish (individual fish = experimental unit) will be individually sedated. Each test fish will be impartially collected from the reference population, placed in an exposure container of a pre-determined concentration of AQUI-S®20E or active control, and timed to handleable. When a fish becomes handleable, it will be immediately transferred to a recovery container of fresh saltwater and allowed to recover. Time-to-handleable, time-to-recovery, and mortality data generated will be analyzed with a two-tailed, exact binomial test.

Lightly sedated: TBD

Collaborations: Collaborators have been not yet been identified to help us conduct trials to evaluate the efficacy of Aqui-S20E to lightly sedate or sedate seawater finfish to handleable. . When identified, these individuals will collaborate by providing test animals, and with experimental setup, study conduct and data collection, and dose verification.

Objective 6: Identify locations/cooperators and conduct trials to demonstrate the effectiveness of Aqui-S20E (10% eugenol) to lightly sedate freshwater salmonids. (Lead: Bowman; Support: Bowker, Carty, Wandelear).

The goal of this objective is provide evidence that Aqui-S20E is effective for lightly sedating freshwater salmonids for extended periods of time (e.g., up to 4 h). As per FDA requirements, studies on at least two different freshwater salmonids will likely need to be completed to demonstrate effectiveness for this claim. The research protocol to describe procedures for conducting studies to evaluate the effectiveness of Aqui-S20E to lightly sedate fish is in development.

Hypothesis. TBD.

Experimental Design. TBD

Collaborations: TBD

Objective 7: Conduct trials in accordance with Good Laboratory Practice regulations to demonstrate the safety of Aqui-S20E (10% eugenol) for lightly sedation of freshwater salmonids. (Lead: Carty; Support: Bowker, Bowman, Wandelear).

The goal of this objective is provide evidence that Aqui-S20E is safe for lightly sedating freshwater salmonids for extended periods of time (e.g., up to 4 h). It is likely that only one study will be required to demonstrate that there is an adequate margin of safety when lightly sedating freshwater salmonids for an extended period of time. The research protocol to describe procedures for conducting studies to evaluate the safety of Aqui-S20E to lightly sedate fish is in development.

Hypothesis. TBD.

Experimental Design. TBD

Collaborations: TBD + contract histologist and quality assurance officer

Objective 8: Conduct trials in accordance with Good Laboratory Practice regulations to demonstrate the safety of Aqui-S20E (10% eugenol) for lightly sedation and sedation to handleable of seawater finfish. (Lead: Bowman; Support: Bowker, Carty, Wandelear).

The goal of this objective is provide evidence that Aqui-S20E is safe for lightly sedating seawater finfish for extended periods of time (e.g., up to 4 hr) and for sedating seawater finfish to handeable. It is likely that at least three studies will be required to demonstrate that there is an adequate margin of safety associated with lightly sedating seawater fish for an extended period of time, and at least three studies will be required to demonstrate that there is an adequate margin of safety associated with sedating seawater fish to handleable. The research protocol to describe procedures for conducting studies to evaluate the safety of Aqui-S20E to lightly sedate fish is in development.

Hypothesis. TBD.

Experimental Design. TBD

Collaborations: TBD + contract histologist and quality assurance officer

Physical and Human Resources:

There are 3.5 Full Time Employee's available (listed on title page) for this project and one histologist, one quality assurance officer (for GLP studies), and at least one Investigator at each study location. In addition to standard equipment needed to clean test tanks, net fish, measure water temperature, dissolved oxygen concentration and other water chemistry parameters, and colorimeters or spectrophotometers to measure concentration of chloramine-T or Aqui-S20E in watter, complete fish rearing facilties are available either at the Bozeman fish Technology Center facilities (plants) or at facilities of project collaborators.

Preparation of Aquaflor medicated feed will be done with a high quality laboratory feed mixer. Analysis of OTC-HCL will be done by USGS Upper Midwest Environmental Sciences Center (LaCrosse WI) under an interagency cooperative agreement or equivalent. Analysis of Aquaflor medicated feed will be done by contract with Eurofins Lancaster Laboratories (Portage MI). Analysis of chloramine-T or Aqui-S20E will be done on-site using spectrophotometric instrumentation.

Project Management and Evaluation - Project team members have sufficient expertise to complete project studies. Researchers for this project are located at one facility, but will be working with collaborators across the country. The project team will meet on a regular basis to discuss upcoming studies, progress, and problems encountered. Study leaders will communicate frequently with collaborators to ensure studies start on schedule; all logistics are in place before starting each study, that data are being collected in a manner that will be acceptable with FDA, and that all data are copied and shipped to the Bozeman Fish Technology Center. Upon arrival, AADAP staff will verify that data are complete and accurate, enter data can enter it electronically, perform data analysis, write the Final Study Report, and submit FSRs to FDA for review. Milestones will be reviewed and updated at the project meetings or electronically to all study participants

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Education/Employment - James Bowker

Education:

Eastern Michigan University, Biological Science, BS, 1982
Eastern Michigan University, Biological Science, MS, 1991

Employment:

2003-present, Research Program Manager, U.S. Fish & Wildlife Service, Aquatic Animal Drug Approval Partnership Program, Bozeman, MT

1994-2003, U.S. Fish & Wildlife Service, Fishery biologist and Assistant NIO Coordinator, Bozeman, MT

1993-1994, Research Chemist, U. S. Geological Survey (formly USFWS) Great Lakes Sciences Center, Ann Arbor MI

1988-1993, Chemist, U. S. Geological Survey (formly USFWS) Great Lakes Sciences Center, Ann Arbor MI

Education/Employment – Dan Carty

Education:

Montana State University, Fish and Wildlife Management, MS, 1985.
University of Maine, Wildlife Management, BS, 1975.

Employment:

1998–present, U.S. Fish and Wildlife Service, Aquatic Animal Drug Approval Partnership Program, Bozeman, MT.

1995-1998, U.S. Fish and Wildlife Service, Creston Fish and Wildlife Center, Kalispell, MT.

1988-1995, U.S. Fish and Wildlife Service, Yellowstone Fishery Assistance Office, Yellowstone National Park, WY.

Education/Employment – Molly Bowman

Education:

Montana State University, Fish & Wildlife Management, BS, 2001

Employment:

11/05–present, Fisheries Biologist, U.S. Fish & Wildlife Service, Aquatic Animal Drug Approval Partnership Program, Bozeman, MT

08/00-11/05, Biological Science Technician (Fisheries), U.S. Fish & Wildlife Service, Aquatic Animal Drug Approval Partnership Program, Bozeman, MT

Education/Employment – Niccole Wandelear

Education:

University of Massachusetts, Amherst, Wildlife & Fisheries Conservation, BS, 1998.
University of Massachusetts, Amherst, Environmental Science, BS, 1998.

Employment:

2006-present, Fishery Biologist, U.S. Fish & Wildlife Service, Aquatic Animal Drug Approval Partnership Program, Bozeman, MT.

2002–2006, Fishery Biologist, U.S. Fish & Wildlife Service, Division of the National Fish Hatchery System, Arlington, VA.

1999–2002, Fishery Biologist, U.S. Fish & Wildlife Service, White River National Fish Hatchery, Bethel, VT.

AADAP Research Team Experience and Accomplishments

AADAP research staff are fishery biologists with specialized skills in experimental design, data collection and analysis, conducting studies in compliance with GLP requirements, conducting research within a strict regulatory framework, and technical writing. Staff have unique experience in developing research study protocols that receive FDA concurrence, developing and adhering to standard operating procedures (SOP) that have been prepared for use of every piece of equipment and procedure to be done during the course of a study, maintaining detailed training records, collecting data according to adequate quality assurance procedures, and

develop detailed final study reports (FSR) for concurrence by FDA reviewers. All field effectiveness studies are conducted according to Good Clinical Practices and target animal safety (TAS) studies are conducted according to Good Laboratory Practices, and AADAP research team staff has extensive experience and training to maintain compliance with GCP and GLP regulations. The AADAP program contracts with an independent Quality Assurance Officer, who is responsible for reviewing every TAS protocol and SOP and inspects an in-life phase component of each TAS study; AADAP research team staff are required to adequately address each comment in the QAO Inspection Report. TAS studies that have contributed to a drug approval are subject to inspection by FDA Field Inspectors who typically spent one week reviewing records to ensure the facility, instrument use logs, training records, and staffing meet GLP requirements and inspect at least one study to ensure that the study had been conducted in compliance with GLP requirements, that SOPs were properly followed, the that the study was conducted according to procedures described in the study protocol. The AADAP program has been inspected twice and passed each inspection. In addition, AADAP staff provided support during an FDA inspection of a TAS study conducted in collaboration with USDA/ARS researchers at the Stuttgart National Aquaculture Research Center. AADAP research staff have submitted approximately 200 effectiveness and 20 TAS FSRs that have contributed to initial approvals or additional approvals of fish drugs or have been sufficient in quality and quantity to complete effectiveness or TAS technical sections.

AADAP research has directly contributed to the following approvals:

1. Aquaflor: control mortality
    a. To control mortality in freshwater salmonids due to coldwater disease
    b. To control mortality in freshwater salmonids due to furunculosis
    c. To control mortality in warmwater fish due to streptococcus iniae
    d. To control mortality in In all freshwater finfish due to columnaris
    e. Use at either 10 or 15 mg florfenicol/kg fish body weight
2. Terramycin 200 for Fish:
    a. To control mortality in freshwater salmonids due to coldwater disease
    b. To control mortality in freshwater *Oncorhynchus mykiss* due to columnaris

AADAP research has directly contributed to new and supplemental approvals – pending sponsor submitting the New Animal Drug Application:

1. Halamid Aqua:
    a. To control mortality in freshwater salmonids due to bacterial gill disease
    b. To control mortality in warmwater fish and walleye due to columnaris.
2. 35% Perox Aid: control mortality/infestation
    a. To control mortality in warmwater fish due to columnaris
    b. To control infestations of *Gyrodactylus* spp. in salmonids
3. Terramycin 200 for Fish
    a. To mark skeletal tissue of all salmonids

AADAP research has completed the following technical sections that will support new fish drug approvals:

1. SLICE:
    a. To effectively control in infestations of *Salmincola californiensis* in Rainbow Trout
    b. To safely treat rainbow trout
2. AQUi-S20E
        a. To effectively sedate freshwater fish to handleable
        b. To safely sedate freshwater fish to handleable
3. 17 alpha methyltestosterone
    a. To effectively produce predominantly male populations of tilapia
    b. To safely treat larval tilapia

Between 2011-2013, AADAP researchers authored or co-authored 17 manuscripts in peer-reviewed publications summarizing results that demonstrated the effectiveness of safety of a variety of fish drugs. During the same period, AADAP researchers authored 15 Drug Research Information Bulletins (an electronically available in-house publication series).

Issues of Concern Statement

Animal Care:
This research involves a variety of fish species and all studies conducted as part of this project will adhere to policies and conditions approved by the Animal Care & Use Committee, Montana State University

Endangered Species:
No endangered species will be used in this project

Environmental Impact Statement:

All experiments of this project will be conducted in laboratories and fish culture facilities of the Service/BFTC or collaborators. The Service has received a categorical exclusion from FDA for completing an Environmental Assessment for use of the drugs, indicating that there will be no potential negative impacts on the environment from use of these drugs.

Human Study Procedure:
No humans will be subjects of the experiments of this project.

Laboratory Hazards:
This research involves working with marginally hazardous materials. All study participants will be familiar with the MSDS for each chemical they may come in contact with

Homeland Security No materials will be used that present a bio-safety hazard in this project.

Intellectual Property Issues There are no protected technologies proposed to be used in the execution of this project plan.

Collaborators

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