Evaluating pre-harvest food safety risks in livestock raised outdoors on diversified small-scale farms in California

By

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DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Epidemiology

in the

OFFICE OF GRADUATE STUDIES

of the

UNIVERSITY OF CALIFORNIA

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2021

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UCDAVIS
EPIDEMIOLOGY
Graduate Group

September 8, 2021

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ACKNOWLEDGMENTS

I am grateful for the mentoring of my advisor Dr. Alda Pires, who encouraged my passion to study agriculture and provided guidance and knowledge throughout my entire graduate school experience. I am also grateful for my mentor Dr. Beatriz Martínez-López, who saw my deep interest in zoonotic diseases and farming and inspired my interest in pigs. Thank you also to the remaining members of my dissertation committee: Dr. Irina Udaltsova for her statistical knowledge and Dr. Joan Rowe for her guidance during the evolution of my research.

Thank you to Dr. Nora Navarro-Gonzalez for spending hours discussing epidemiology, foodborne pathogens and pigs with me while driving the back roads of California to collect farm samples. Thank you also to other co-authors who provided time, dedication and knowledge to improve my dissertation chapters: Dr. Jaber Belkhiria, Dr. Erin DiCaprio, Dr. Richard V. Pereira, La'Chia Harrison, Dr. Carl Basbas, and Dr. Thais Ramos. Deep gratitude for all my professors at UC Davis including Dr. Maurice Pitesky, Dr. Philip Kass, Dr. Dr. Robert Atwill, Dr. Janet Foley, Dr. Bruno Chomel, Dr. Chris Barker, Dr. Brett McNabb, Dr. Rebecca Schmidt, Dr Mary Christopher, Dr. Bradley Pollock, Dr. Theresa Keegan, Dr. Maria Marco, Dr. Ellen Gold, Dr. Irva Hertz-Piciotto, Dr. Laurel Beckett, Dr. Heejung, Dr. Woutrina Smith and Dr. David Rocke.

I want to express my heartfelt gratitude to my friends at UCD - thank you for your friendship, humor and fun adventures during graduate school (in alphabetical order): Amlan Aggrawal, Abhi Bari, Dr. Jerome Baron, Dr. Karen Holcomb, Dr. Wendi Jackson, Gad Keidar, Dr. Ester Kukielka, Dr. Kyuyoung Lee, Dr. Hanqi Luo, Dr. Gaby Meier, Brandon Merritt, Dr. Nora Navarro-Gonzalez, Dr. Kathleen O'Hara, Seanchan Owen, Dr. Pascale Stiles, Dr. Gema Vidal, Dr. Xiao Wei and Dr. Tada Yatabe. I could not have completed my PhD without your support.

Thank you also to the many farmers who graciously volunteered to participate in our studies: for all of you who need to remain anonymous due to privacy concerns, thank you so much for believing in my research projects and trusting me to conduct field work on your farms. And thank you to all USDA-WS, USDA-APHIS, CDFW and CDFA staff who spent time working with me to cultivate projects involving feral pigs, including Dr. Dana Nelson, Dr. Ben Gonzalez, Dr. Hector Webster and Dr. Rebecca Mihalco.

Special heartfelt appreciation and gratitude for my husband Eric Patterson, who has supported and encouraged me with kindness and love throughout my entire graduate school program. He also provided technical support (making sampling tools, weed-whacking, etc.) and conducted field work with me. I would not have finished my PhD without you - you are my foundation and love.

Thank you all!

Dissertation Abstract

The increasing number of diversified small-scale farms (DSSF) and outdoor-raised livestock in California and nationwide, reflects growing consumer interest and demand for organic or sustainably-produced local foods, including humanely-raised animal products such as meat and eggs. However, there is a lack of research evaluating the unique agricultural management practices of DSSF and how these farming systems may involve risk factors that affect the transmission of foodborne pathogens in the food supply.

Diversified farms are often small-scale and raise a combination of livestock and produce, or multiple livestock species with the intent of selling specialized animal products directly to consumers. However, livestock are reservoirs for foodborne pathogens like Shiga toxin-producing *Escherichia coli* (STEC). Additionally, raising pigs outside provides an opportunity for contact with feral pigs, which harbor zoonotic and foodborne pathogens. STEC remains one of the major causes of foodborne outbreaks in the US and STEC outbreaks associated with DSSF are most likely underreported

The overall goal of this dissertation focused on evaluating the pre-harvest food safety risks on DSSF in California: Chapter 1 estimated the prevalence of STEC in cattle, goats, sheep and pigs raised on DSSF and evaluated risk factors associated with the prevalence of STEC using a multilevel logistic regression model. Chapter 2 used a species distribution modeling method Maximum Entropy (MaxEnt) to predict suitable habitat for feral pigs in California. Then the MaxEnt prediction map was overlapped with the location of over 300 outdoor-raised pig operations (OPO) in California to create a risk map, identifying areas most at risk for disease transmission between these two growing swine populations. In the past few decades, California has experienced an increase of feral pigs and a resurgence of outdoor-based domestic pigs and

this trend has implications for pathogen transmission in the wildlife-livestock interface. The feral pig–outdoor-raised pig risk map built in Chapter 2 identified counties to target for a STEC prevalence study in Chapter 3. Fecal samples from both feral pigs and domestic pigs raised outdoors were collected in counties that had a higher risk for contact between feral pigs and domestic swine raised outdoors. The overall goal of Chapter 3 included assessing the prevalence of STEC in those counties as well as using a multilevel logistic regression model to assess risk factors associated with the presence of STEC in fecal samples.

Although consumers perceive small-scale farms or outdoor-raised meat as safer or more natural, these three studies together demonstrated that livestock raised outdoors on small-scale farms are reservoirs for STEC and indicated the need for more studies to ascertain risk factors of foodborne pathogens on DSSF. As the number of DSSF farms continues to grow, evaluating risk factors and management practices that are unique to these small operations will help identify risk mitigation strategies and develop extension outreach materials to keep food safe from farm to fork and protect California's agricultural economy.

Dissertation Introduction

The increasing number of diversified small-scale farms (DSSF) and outdoor-raised livestock (i.e., grass-fed, pasture-raised) in the United States (US), reflects growing consumer interest and demand for sustainably-produced or organic local foods, including animal products such as meat and eggs.(1)(2) However, there is a lack of research evaluating the unique agricultural management practices of DSSF and if these types of farming operations involve risk factors that affect the transmission of foodborne pathogens in the food supply.

California is the top agricultural production state in the US with annual sales over $50 billion from 69,900 total farms.(3)(4) California is also the sole producer in the US (defined as producing at least 99% of a crop) of 17 crops including figs, artichokes and almonds.(3) Additionally, California leads organic sales, accounting for 40% of all organic crop production and 18.16% (3,012/16,585) of United States Department of Agriculture (USDA) certified organic farms.(3)(5) Many organic farms are small-scale and diversified and these type of DSSF sell food directly to consumers through marketing channels, such as farmers markets or Community Supported Agriculture (CSA) programs.(6)(7) To suit the unique characteristics of California's diverse, and within interior valley regions, year-round growing environment, we adjusted the USDA-ERS definition of "small-scale farm" to encompass operations that gross less than $500,000 annually and market directly to consumers through farmers markets, farm stands, CSAs, etc.(8-10) DSSF are defined as those operations that grow a combination of livestock and specialty crops or raise multiple livestock species with the intent of selling sustainably-raised animal products (e.g., pasture-based) directly to consumers. Some diversified farms integrate livestock and crop production (i.e., integrated crop-livestock or mixed crop-livestock farms) by using their animals to graze crop residues or cover crops before planting with fresh market crops.

(1)·(11) Grazing enhances soil fertility, recycles farm nutrients and animals provide another source of revenue through fiber or food products.(1)(12)(13)

Many consumers perceive small-scale farms or outdoor-raised livestock as more natural and safer than food grown on large-scale conventional farms or meat animals raised in confinement systems, but animals naturally harbor foodborne pathogens that can cause severe human illness, like *Salmonella* spp*., Campylobacter* spp. and Shiga toxin-producing *Escherichia coli* (STEC).(2)(14-19) For instance, a study by Patterson *et al* (2018) calculated a 4.17% prevalence of STEC in sheep raised on a diversified organic farm in California.(20) Animals are intermittent shedders of enteric pathogens and shedding may increase under certain conditions, such as during periods of stress (e.g., transportation) or due to husbandry practices (e.g., diet, stocking density).(21)(22) Many foodborne pathogens can exist in the soil for extended periods of time and can be transmitted to humans through direct contact with feces or animals, or indirect contact with a contaminated environment or through ingestion of produce, meat or water.(23)(24)

STEC is consistently one of the major pathogens involved in foodborne outbreaks in the US.(25-27) Vegetables and fruit consumed raw, including spinach, tomatoes and melons, are especially considered high-risk foods.(28-31) In the summer of 2011, a small family farm u-pick berry operation was the center of an *E. coli* O157:H7 outbreak when strawberries were contaminated by wild deer feces.(32) Six of the fifteen cases were hospitalized and two people died. More recently, several nationwide outbreaks of *E. coli* O157:H7 have occurred through consumption of romaine lettuce, including one outbreak traced back to California farms and linked to cattle grazing upstream from the lettuce fields.(33) STEC outbreaks associated with DSSF might be underreported, due to their smaller volume of sales compared to large farms. A

study by Harvey *et al* (2016*)* identified six STEC outbreaks connected with organic agricultural operations between 1992-2014.(25) All these foodborne outbreaks underscore the need to conduct prevalence studies of foodborne pathogens on DSSF.

Swine are especially a livestock species of concern because they are reservoirs for zoonotic diseases like swine influenza *and* brucellosis and foodborne pathogens like STEC and *Campylobacter* spp. (14)(16-19)(34-37) Although most swine production in the US occurs inside buildings with high levels of biosecurity, the US is currently experiencing a resurgence of outdoor-based swine operations, due to consumer demand for sustainably-raised animal products.(38-40) Still primarily considered a niche production system in the US, outdoor-raised pig operations (OPO) (e.g., backyard producers, commercial meat operations) are numerous and broadly distributed within California. The USDA National Animal Health Monitoring System (NAHMS) swine report defined "small-enterprise operations" as those raising fewer than 100 pigs.(41) Approximately 68.8 - 78.9% of the small operations included in this nationwide survey raised domestic pigs with some level of outside access.(41)

A challenge in raising domestic pigs outdoors is the increasing risk of their directly or indirectly interacting with wildlife like feral pigs, and a subsequent potential increase for pathogen sharing, especially as feral pig abundance and distribution grows throughout the US.(37)(42-47) Feral pigs are considered an invasive species as they only need water, food and shrub cover to survive, can double their population in four months and are difficult to eradicate.(34)(48)·(49) Moreover, if an area contains favorable habitat for feral pigs, (e.g., agricultural areas, oak forests) then their population numbers can be maintained or increase over time.(50) They also have the widest geographic distribution and one of the broadest habitat ranges of any large mammal except humans.(34) The wide distribution of feral swine is in part

due to their ability to adapt to many ecological habitats and their opportunistic omnivore diet.(34) California has one of the largest and widest distributions of feral pigs.(51)(52)

Feral pigs in the US are a mix of introduced Eurasian wild boars, which are native to Asia and Europe, and domestically-raised pigs turned feral. Feral and domestic pigs share the same genus and species (*Sus scrofa*) and therefore can share pathogens.(45)(49)(53)(54) As early as 2005, Corn *et. al* described the disease implications of expanding feral pig populations in the US, because they can serve as a vehicle for pathogen transmission to domestic pigs, and they could play a significant role in the transmission and maintenance of transboundary animal diseases (TBD), that may be introduced or re-introduced to North America (e.g., African swine fever (ASF), classical swine fever).(46)(43) Other studies have also reported the disease risks from the expanding distribution of feral pigs in the US, including their future role as spreaders of TBD like ASF, which was recently found in the Dominican Republic, a mere 700 miles from Florida, US.(47)(55-57) Additionally, eradicated diseases in indoor-pig herds (e.g., pseudorabies, brucellosis) have been documented in feral swine populations in California, for example, feral pig samples collected by the California Department of Fish and Wildlife (CDFW) from 1978-2013 showed feral pigs testing positive for *Brucella suis*, *Leptospira* spp. and swine influenza virus (Gonzales, unpublished).(42)(44) Contact between feral pigs and outdoor-raised pig herds increases the risk for the transmission of these diseases in domestic swine.(42)(44)(58)(37)

Many studies have reported that feral pigs (also known as wild hogs, wild boars, etc.) maintain and transmit zoonotic and foodborne pathogens; however, only a small subset of these studies focused on the risk of pathogen sharing between feral pigs and outdoor raised pigs. (35)(43)(46)(56)(59) Transmission of pathogens between feral pigs and outdoor raised pigs has been documented in the US. (56)(46)(60) For example, a 2016 human brucellosis case on a New

York State farm began with a feral pig infecting domestic pigs reared outdoors.(60) Swine sold from this index farm led to *Brucella suis* positive domestic swine in nine other herds in multiple states.(60) Additionally, one swine brucellosis case each in Texas, Iowa and Georgia in 2005 also involved domestic swine being exposed to feral pigs through inadequate biosecurity or wildlife controls.(46)

Feral pigs are known to forage on farmland and some California farmers and ranchers regularly experience feral pig intrusions in their crop fields and/or contact between outdoor-raised pigs and feral pigs.(49) According to studies conducted in California and Texas, contact has been documented between feral pigs and outdoor-raised pigs.(57)(56) A 2012 spatial study by Wyckoff *et al* (2012) reported that feral pigs are attracted to agricultural habitats as food sources, which may facilitate pathogen transmission to livestock raised outdoors and humans or contaminate crops.(59) The authors assessed habitat and movement of feral swine within 10 miles (i.e., 16.10km) of outdoor domestic pig operations in Texas and calculated that at least 50% of these facilities were surrounded by suitable feral pig habitat.(59) Another Wyckoff *et al* (2009) study assessed the disease transmission risk of feral pigs near domestic pigs facilities in Texas. (56) This 2009 study used GPS collars to quantify contact between feral and domestic pigs and detected evidence of direct contact, as well as antibodies for the same diseases (i.e., brucellosis, pseudorabies) in both swine groups.(56) They concluded that feral swine are an increasing risk for the reintroduction of eradicated diseases as well as emerging TBD, especially for operations that allowed domestic swine outdoor access, as male feral pigs are attracted to female pens.(56) International studies have also assessed the risk of disease transmission at the wild boar-domestic pig interface.(61-63) All these findings highlight the need for further

investigation to identify high-risk areas for disease spread in the event of a future disease outbreak in California or nationwide.

My overall thesis goal entailed evaluating the risk of STEC on DSSF and the risk of disease transmission in the spatial overlap of feral pigs and domestic swine raised outdoors in California. In Chapter 1, we collected fecal samples from cattle, goats, sheep and pigs raised on DSSF to estimate the prevalence of STEC on these unique operations. I used a multilevel logistic regression model to assess the association between risk factors and STEC presence in fecal samples. In Chapter 2, I used a species distribution modeling (SDM) method Maximum Entropy (MaxEnt) to predict suitable habitat for feral pigs in California. Then I overlapped this MaxEnt model with the location of over 300 OPO in California to create a risk map that identified areas most at risk for disease transmission between these two growing swine populations. The increasing potential for contact between domestic swine raised outdoors and feral pigs provides an opportunity for the widespread transmission of diseases throughout California, as each pig could serve as a link in the transfer of pathogens between wildlife, livestock and humans. Additionally, the transmission of diseases to domestic pigs raised outside could negatively impact the sustainability of California's agriculture economy. In Chapter 3, I designed a study to evaluate the prevalence of STEC in six counties at highest risk for contact between feral pigs and OPO, based on the risk map built in Chapter 2. I collected fecal samples from both feral pigs and domestic pigs raised outdoors and used a multilevel logistic regression model to assess risk factors associated with the presence of STEC in samples. The results of these last two chapters fill critical information gaps regarding the epidemiology of STEC harbored in outdoor-raised pigs on DSSF located near suitable feral pig habitat.

Chapter 1

Prevalence and risk factors of Shiga toxin-producing *Escherichia coli* in livestock raised on diversified small-scale farms in California

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Abstract

The increasing number of diversified small-scale farms (DSSF) that raise outdoor-based livestock in the United States, reflects growing consumer demand for sustainably-produced local foods. Diversified farms are most often small-scale and raise a combination of livestock and numerous produce varieties, or multiple livestock species, with the intent of selling sustainably-raised animal products directly to consumers. However, there is a lack of science-based information characterizing the risk factors for transmission of foodborne pathogens, such as Shiga toxin-producing *Escherichia coli* (STEC), in livestock raised on DSSF. Livestock are asymptomatic reservoirs and intermittent shedders of enteric pathogens.

The aim of this study is to a) describe the unique characteristics of DSSF, b) estimate the prevalence of STEC in livestock raised on DSSF and c) evaluate the association between risk factors and the presence of STEC in livestock raised on DSSF located in California.

During 2015-2016, we conducted a repeated cross-sectional study to test for STEC in fecal samples collected from livestock raised on DSSF. Livestock species sampled in this study included dairy and beef cattle, dairy and meat goats, pigs and sheep. A 41-question questionnaire was administered to farm owners. Prevalence of STEC was estimated and generalized linear mixed models were used to assess the association between STEC presence in fecal samples and risk factors.

Sixteen farms participated in our study and were located in four regions of California. Overall STEC prevalence was 13.62% (76/558) and farm-level STEC prevalence ranged from 0% to 30%. Positive STEC samples were detected in all sampled livestock species. Significant variables in the mixed effect logistic regression model included daily maximum temperature (OR = 0.95; $CI_{95\%}$: 0.91-0.98), livestock sample source (cattle (OR = 4.61; $CI_{95\%}$: 1.64-12.96) and

sheep (OR = 5.29; $CI_{95\%}$: 1.80-15.51)), multiple species shared the same barn (OR = 6.23; $CI_{95\%}$: 1.84-21.15) and livestock had contact with wild areas (OR = 3.63; $CI_{95\%}$: 1.37-9.62).

Identification of STEC serogroups of public health concern in this study indicate the need for mitigation strategies to keep food safe by evaluating the risk factors and management practices that lead to the spread and prevalence of foodborne pathogens in a pre-harvest environment on DSSF.

Introduction

The increasing number of diversified small-scale farms (DSSF) that raise outdoor-based (i.e., grass-fed, pasture-raised) livestock in the United States (US), reflects growing consumer demand for sustainably-produced local foods, including animal products such as meat and eggs.(1)(2) (9)(64-66) California is the top producer of agricultural products in the US and also leads the country in organic food sales, which includes products from DSSF (64)(10) However, there is a lack of science-based information characterizing the risk factors associated with the prevalence of foodborne pathogens, such as Shiga toxin-producing *Escherichia coli* (STEC), in livestock raised on DSSF.

Diversified farms are most often small-scale and raise a combination of livestock and numerous types of produce or multiple livestock species, with the intent of selling specialized (e.g., organically-grown, pasture-based) animal products directly to consumers.(66) Some diversified farms integrate livestock and crop production (i.e., integrated crop-livestock farms) by using their animals to graze crop residues or cover crops before planting a field to fresh produce.(1)(11) Grazing improves soil fertility and provides farm owners with another source of revenue through fiber or meat products.(1)(12)

Many consumers perceive produce grown on small-scale farms and/or meat raised on pasture as more "natural" or safer (i.e., chemical-free or pesticide-free) than food grown on large-scale conventional farms or meat animals raised in confinement systems.(2)(25)(15) However, livestock are asymptomatic reservoirs for foodborne pathogens and without adequate mitigation strategies, these pathogenic microorganisms may enter into the food supply.(14)(67-73) Livestock are intermittent shedders of enteric pathogens and shedding may increase under certain conditions, such as during periods of stress (e.g., transportation), due to certain management

practices (e.g., changes in diet, high stocking rate) or seasonally.(74) Foodborne pathogens survive in the soil for extended periods of time and can spread to humans directly through contact with livestock or indirectly via contaminated food (e.g., produce, meat, eggs) or water.(23)(75-77) For instance, cattle grazing uphill from a produce field was likely the causative factor for the 2019 *E. coli* O157:H7 romaine lettuce outbreak.(27) STEC remains one of the top enteric pathogens associated with foodborne outbreaks in the US.(30) The top seven STEC O-serogroups that cause the most severe illness in humans (e.g., O157:H7, O26, O103, O111, O121, O145, O45) have been traced to consumption of produce consumed raw, such as spinach, tomatoes and melons. (74)(78)(79) Fresh produce consumed raw (e.g., tomatoes, melons), which has been contaminated by livestock or wildlife feces containing STEC, can become a vehicle for these pathogens to enter the food supply. (29)(28)(80-82)

The aim of this study is to a) describe the unique characteristics of DSSF, b) estimate the prevalence of STEC in livestock raised on DSSF and c) evaluate the association between risk factors and the presence of STEC in livestock raised on DSSF located in California.

Materials & Methods

Study design and farm enrollment

During 2015-2016, we conducted a repeated cross-sectional study in Northern and Central California to test for *Campylobacter* spp., *Salmonella* spp. non-O157 STEC and *E. coli* O157:H7 in fecal samples collected from livestock raised on DSSF. This current manuscript reports that STEC and *E. coli* O157:H7 results.(14) Enrollment criteria for participating DSSF was based on USDA-ERS definition of small-scale farms (9)(83), but adjusted to fit the unique growing conditions of California operations: 1) small- to medium-scale farm (i.e., poultry producers raising <1,000 birds per year or livestock producers with an annual gross-sales

<$500,000 and that raised a maximum of 500 goats/sheep and/or 100 cattle and/or 100 pigs); 2) raised a diversity of crops and/or multiple species of livestock and poultry; 3) marketed farm products directly to consumers, for instance through farmers markets or a Community Supported Agriculture (CSA) program; and 4) willingness to participate. We enrolled farms from four regions closest to our university: Shasta Cascade, North Coast, Central Valley and Central Coast.(84) Farm recruitment occurred through personal communications, announcements at agriculture outreach seminars and contact at farmers markets or agricultural fairs.

Sample collection

Farms were visited twice between May 2015 and June 2016, once during each of the following periods: summer/autumn or winter/spring, which reflect California growing seasons and the seasonality of STEC shedding.(14) Livestock species sampled in this study included dairy and beef cattle, dairy and meat goats, pigs and sheep. Sample sizes were calculated using Epitools based on the total number of animals on each farm, with an assumed STEC prevalence of 5% and 10% precision error, stratified by each livestock species.(85) Individual fresh fecal samples were collected from the ground. Samplers wore gloves and placed approximately 50-100 grams of feces into each sterile whirl-pak bag (Nasco, Modesto, CA). Bags were immediately placed into plastic coolers containing ice packs, transported to the laboratory at the end of the sampling day and most samples processed within 24 hrs.

E. coli *O157:H7 and non-O157 STEC culture and PCR*

STEC was isolated from fecal samples as described previously with modifications.(74) In brief, 10 grams of fecal material was placed in 90 ml Tryptic Soy Broth (TSB; Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and homogenized before and after. Samples were then incubated for 2 hrs at 25°C with 100 rpm agitation, followed by 8 hrs at 42°C with 100

rpm agitation, and held overnight at 6°C, using a Multitron programmable shaking incubator (Eppendorf, Hauppauge, NY). For detecting *E. coli* O157:H7, immunomagnetic separation (IMS) using Dynal anti-*E. coli* O157 beads (Invitrogen/Dynal, Carlsbad, CV) was performed on TSB enrichment broths with the automated Dynal Bead Retriever (Invitrogen) per the manufacturer's instructions. After incubation and washing, 50 µL of the resuspended beads were plated onto Rainbow agar O157 (Biolog, Hayward, CA) with novobiocin (20 mg/L) and tellurite (0.8 mg/L) (MP Biomedicals, Solon, OH). Fifty µL of the resuspended beads were also plated onto MacConkey II Agar using sorbitol supplemented with potassium tellurite (2.5 mg/L) and Cefixime (0.05 mg/L) (CT-SMAC); plates were streaked for isolation and incubated for 24 hrs at 37°C. Suspect *E. coli* O157:H7 isolates were confirmed using traditional PCR for the *rfbE* gene.(86)

To detect non-O157 STEC, 1 mL of pre-enrichment broth was incubated in mEHEC selective media (Biocontrol, Bellevue, WA) for 12 hrs at 42°C followed by plating and incubating on Chromagar STEC (DRG International Inc., Springfield, NJ). Up to 8 presumptive STEC positive colonies were confirmed for the presence of *stx1* and/or *stx2* genes by real-time PCR. (74) Confirmed STEC isolates were then characterized for virulence genes (*stx1*, *stx2*, *eaeA*, *hlyA* and *ehxA*) using conventional PCR.(86) After PCR testing, one colony from each positive sample was submitted to the Pennsylvania State University *E. coli* Reference Center to confirm O-serogroups.(74)(87)

Farmer questionnaire and environmental factors

A 41-question questionnaire, consisting of mostly closed-ended questions, was administered to farm owners at the end of the study period. The questionnaire included sections regarding farm demographics, animal health, farm management practices, and water sources

(questionnaire provided upon request). Variables analyzed for model building included risk factors from the farmer questionnaire, sample day factors (e.g., temperature) and variables that were created using known information about each farm, for instance, whether a farm raised multiple types of livestock or if they integrated livestock within produce fields before planting. Variables from the questionnaire included whether farmers allowed different livestock species to share the same barn and if livestock had contact with wild areas (e.g., streams, forest, wetlands). Weather data from the nearest California Irrigation Management Information System (CIMIS) weather station (http://www.cimis.water.ca.gov) within a similar microclimate, provided environmental factors for possible model inclusion (e.g., average relative humidity, daily maximum temperature).(88) Also, USDA plant hardiness zones, which are based on the average annual minimum winter temperature, were included as a proxy for the many microclimates in California (Figure 1.1).(89) Only three zones were necessary to categorize our participating farms: zone 7b (-15 to -12.2 ºC), 9a (-6.7 to -3.9 ºC) and 9b (-3.9 to -1.1 ºC).

Figure 1.1: USDA plant hardiness zone map used as a proxy for the various microclimates found in California during generalized linear model building to assess the association between risk factors and the presence of Shiga toxin-producing *Escherichia coli* in fecal samples collected from diversified small-scale farms in California between May 2015 and June 2016. USDA Zone map can be found online: https://planthardiness.ars.usda.gov

Statistical data analysis and model building

Descriptive statistics, (e.g., mean, range) were calculated for all data. STEC was estimated for the overall study and per livestock species (number of positive samples divided by total number of samples collected per category, for instance swine). Generalized linear mixed models were used to assess the association between STEC presence in fecal samples and risk factors. The binary outcome of interest was whether each fecal sample was STEC positive or negative. Univariate analysis assessed the distribution of variables. During bivariate analysis, variables with low variability, small cell sizes (<5), or large standard errors were either modified, collapsed if appropriate, or discarded from model building.(90)(91) Correlation of numeric variables was measured with Spearman's rank correlation coefficient; those variables that were correlated 0.80 or more were highlighted during the model-building phase to evaluate for multicollinearity issues. To identify possible confounders, each variable was evaluated using a directed acyclic graph (DAG) and then added to the model to determine whether the variable affected the odds ratios of the other variables by more than 10%.(92)

The glmer function was used from the lme4 package in R to build models, with farm added as a random effect to account for possible farm-level clustering effects when analyzing individual samples.(93) Manual two-way stepwise variable selection was employed for model building. Variance inflation factors (VIF) identified possible multicollinearity and variables in the model that had a VIF over 5 were assessed for removal. Top models were compared, and a final model chosen based on the lowest Akaike Information Criterion (AIC), smallest deviance, relative to the other models. Intraclass correlation (ICC). Diagnostic plots from the DHARMa package in R were used to assess the final model and included fitted vs binned residuals, a Q-Q plot and the Kolmogorov-Smirnov test statistic.(94) Odds ratios and 95% confidence intervals

($CI_{95\%}$) were calculated for variables in the final model. All data analysis was performed using R Statistical Software (i.e., R) version 1.4.1036 ©.(95)

Results

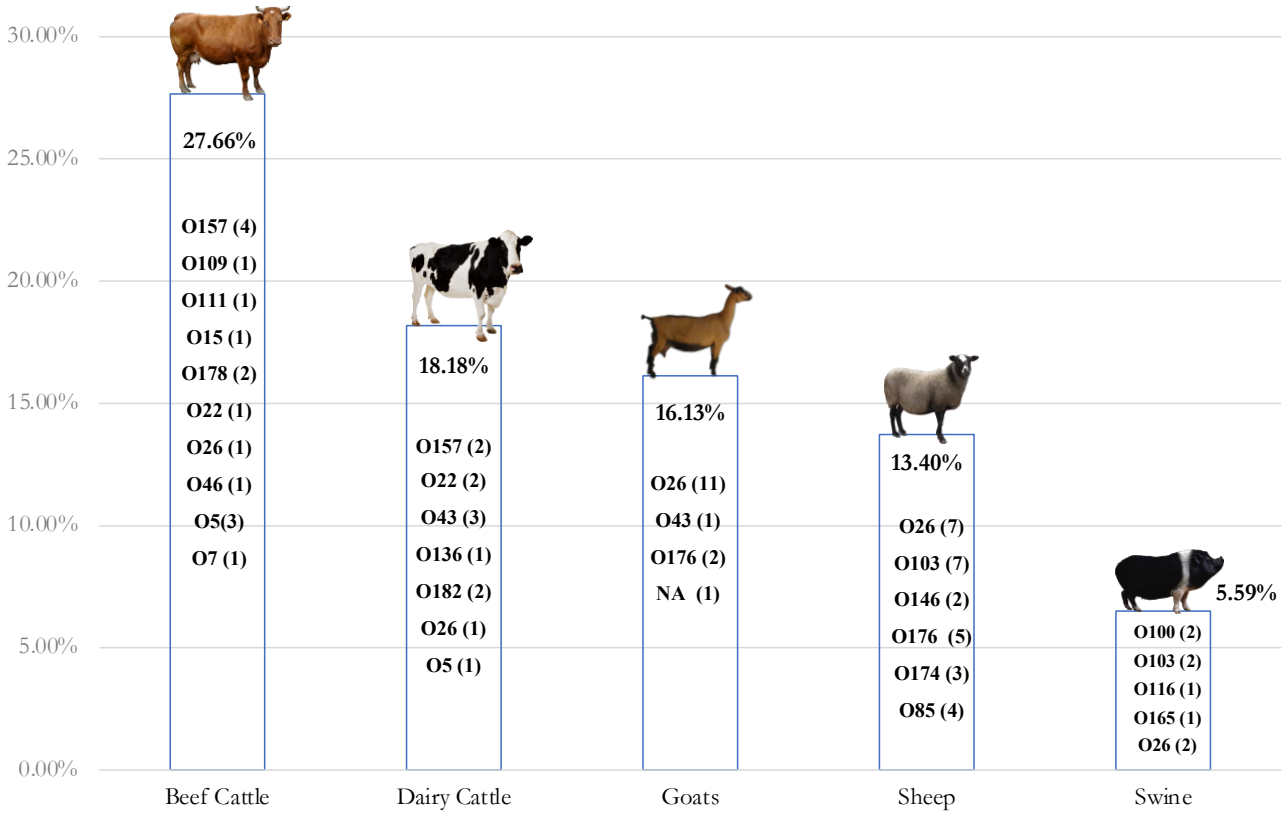
Study participants

Sixteen farms participated in our study and were located in four regions of California: Central Valley (n=10), Central Coast (n=3), Shasta Cascade (n=2) and the North Coast (n=1). Four of the sixteen farms (25.00%) raised livestock only, nine farms (56.25%) raised a combination of produce and livestock and used the animals to graze crop fields, and three produce farms (18.75%) rented sheep seasonally to graze cover crops and did not raise any other livestock or poultry. Four farms were not organic (25.00%), 56.25% (9/16) were certified organic and 18.75% (3/16) used organic practices but were not certified by a third party.

STEC prevalence

A total of 558 fecal samples were collected from 16 farms. Overall STEC prevalence was 13.62% (76/558; $CI_{95\%}$: 10.88 - 16.75%) Farm-level STEC prevalence ranged from 0% - 30%; however, 37.50% (6/16) of farms had no positive samples. Of the 62.50% (10/16) of farms with positive STEC samples, the mean prevalence was 17.24%, with a median of 16.73%. Positive STEC samples were detected in all sampled livestock species. STEC prevalence and the count of each O-serogroup per livestock species are shown in Table 1.1. Beef cattle had the highest STEC prevalence at 27.66% (13/47: $CI_{95\%}$: 15.62 - 42.64%) and second highest were dairy cattle 18.18% (12/66; $CI_{95\%}$: 9.76 - 21.61%), with a 22.12% prevalence for all cattle combined. Goats had the next highest prevalence at 16.13% (15/93; $CI_{95\%}$: 9.32 - 25.20%), and next sheep 13.40% (28/209; $CI_{95\%}$: 9.09 - 18.78%). Swine samples had the lowest prevalence: 5.59% (8/143; $CI_{95\%}$: 2.45 - 10.73%). *E. coli* O157:H7 was found only in cattle, for a prevalence of 5.31% (6/113).

Table 1.1: Shiga toxin-producing *Escherichia coli* prevalence (%) and O-serogroup* (count) per livestock species for n=76 positive samples collected between May 2015 and June 2016 from 16 diversified small-scale farms in California.



** One O-serogroup was untypeable. Three positive samples contained two serogroups each.*

Of the 76 positive STEC fecal samples, 73 were non-O157 STEC, with three samples being positive for both *E. coli* O157:H7 and a non-O157 STEC (all three from beef cattle), for a total of 79 O-serogroups. O26 was the most prevalent O-serogroup, accounting for 27.85% (22/79) of positive isolates (Table 1.2). One STEC positive sample was unable to be serotyped. Virulence factors for the 79 O-serogroups are listed in Table 1.3. *Stx2* was identified in 16.46% (13/79) of O-serogroups, *stx1* was identified in 69.62% (55/79) and 13.92% (11/79) contained both *stx1* and *stx2*. All six O157:H7 positive isolates contained virulence factors *stx2*, *ehxA*, *hlyA* and *eaeA*, but not *stx1*.

Table 1.2: O-serogroups (count and percentage) identified in 79* Shiga toxin-producing *Escherichia coli* positive isolates collected from diversified small-scale farms in California, between May and to June 2016, compared to CDC's list of the top 7 serogroups of concern for public health from the 2016 National Shiga toxin-producing *Escherichia coli* Surveillance Annual Report.

CDC Rank	O-Serogroup	#	%
1	O157	6	7.59%
2	O26	22	27.85%
3	O103	9	11.39%
4	O111	1	1.27%
5	O121	0	0.00%
6	O45	0	0.00%

** Includes three samples classified as O157:H7 that contained a second non-O157 serogroup.*

Table 1.3: Virulence genes discovered in 79 O-serogroups from 76 positive Shiga toxin-producing *Escherichia coli* samples[τ] collected from 16 California diversified small-scale farms, between May 2015 - June 2016, separated by livestock species, O-serogroup and genes.

Source	# isolates*	O-serogroup	*ehxA*	*hlyA*	*eaeA*	*stx2*	*stx1*
Beef cattle	1	O109				+	
Beef cattle	1	O111	+	+	+	+	+
Beef cattle	1	O15				+	
Beef cattle	4	O157	+	+	+	+	
Beef cattle	2	O178	+	+		+	
Beef cattle	1	O22	+	+		+	+
Beef cattle	1	O26	+	+	+		+
Beef cattle	1	O46	+	+		+	+
Beef cattle	3	O5	+	+	+		+
Beef cattle	1	O7					+
Dairy cattle	1	O136	+	+		+	
Dairy cattle	2	O157	+	+	+	+	
Dairy cattle	2	O182	+	+	+		+
Dairy cattle	2	O22	+	+		+	+
Dairy cattle	1	O26	+	+			+
Dairy cattle	3	O43	+	+		+	+
Dairy cattle	1	O5	+	+	+		+
Goat	2	O176	+	+			+
Goat	11	O26	+	+	+		+
Goat	1	O43	+	+		+	+
Goat	1	NA	+	+	+		+
Sheep	7	O103	+	+	+		+
Sheep	2	O146	+	+		+	+
Sheep	3	O174					+
Sheep	5	O176	+	+			+
Sheep	7	O26	+	+	+		+
Sheep	4	O85	+	+			+
Swine	2	O100				+	
Swine	2	O103	+	+	+		+
Swine	1	O116		+	+		+
Swine	1	O165	+	+			+
Swine	2	O26	+	+	+		+

*Number of isolates containing the same O-serogroup and virulence genes.

τ One O-serogroup was untypeable.

Risk Factor Analysis

Of the 16 participating farms, two were not included in model building, as their questionnaires were not completed, leaving a total of 502 fecal samples for model building. The mean, median and range of selected numeric variables assessed for inclusion during model building are shown in Table 1.4. Farms in this study ranged from two to 500 acres and had been farming two to 30 years. Stocking rate was calculated by dividing the total number of livestock, excluding poultry, by the total number of farm acres (i.e., number of animals / number of farm acres). Selected categorial variables, stratified by whether they were STEC positive or negative are presented in Table 1.5.P -values were reported for chi-square test or Fisher's Exact test if cell sizes were less than five. For instance, 28.99% (20/69) of positive samples came from farms that mixed livestock species within a barn, whilst only 15.70% (68/433) of negative samples came from farms with shared barns (p-value 0.012). Moreover, 72.46% (50/69) of positive samples were from farms that allow livestock to have contact with bordering wild areas (p-value 0.026).

Table 1.4: Mean, median and range (i.e., minimum and maximum) of selected numeric factors assessed for model building and collected during a cross-sectional study conducted from 2015-2016 on 14* diversified small-scale farms in California.

Description	Mean	Median	Minimum	Maximum
Number of farm acres	88.93	29	2	500
Average relative humidity (%)	52%	45%	23%	95%
Daily maximum temperature (ºC)	28.32ºC	29.00ºC	11.70ºC	39.80ºC
Daily minimum temperature (ºC)	10.85ºC	11.60 ºC	0.40ºC	21.40ºC
Density (# animals/acre)	3.11	1.48	0.20	13.75
Soil temperature (ºC)	19.41ºC	20.60ºC	3.80ºC	28.70ºC
Total # chickens per farm	1,984	100	0	21,500
Total # cattle (beef or dairy) per farm	8	0	0	47
Total # goats (dairy or meat) per farm	8	5	0	31
Total # sheep per farm	37	0	0	181
Total # pigs per farm	36	10	0	325
Number of years farm has been in operation	10.29	7	2	30

**Two of the total 16 participant surveys were not completed.*

Table 1.5: Bivariate analysis for selected categorical variables stratified by positive (n=69) or negative (n=433) Shiga toxin-producing *Escherichia coli* status. Samples were collected during a cross-sectional study from 2015-2016 on 14* diversified small-scale farms in California.

Description	Levels	STEC Negative ct (%)	STEC Positive ct (%)	p-value (chi-sq)
California regions based on USDA climate zones [a.]	7b	87 (20.09%)	25 (36.23%)	0.003*
	9a	206 (47.58%)	20 (28.99%)	
	9b	140 (32.33%)	24 (34.78%)	
Different livestock species share the same barn	No	365 (84.30%)	49 (71.01%)	0.012*
	Yes	68 (15.70%)	20 (28.99%)	
Farm rotates different animals within the same field	No	140 (32.33%)	27 (39.13%)	0.329
	Yes	293 (67.67%)	42 (60.87%)	
Livestock were allowed contact with wild areas	No	184 (42.49%)	19 (27.54%)	0.026*
	Yes	249 (57.51%)	50 (72.46%)	
Is the farm certified organic [b.]	No	129 (29.79%)	19 (27.54%)	0.073
	Not certified	125 (28.87%)	29 (42.03%)	
	Yes	179 (41.34%)	21 (30.43%)	
Does farm raise swine	No	82 (18.94%)	16 (23.19%)	0.507
	Yes	351 (81.06%)	53 (76.81%)	
Species of collected sample	Cattle	88 (20.32%)	25 (36.23%)	0.002*
	Goats	78 (18.01%)	15 (21.74%)	
	Sheep	132 (30.48%)	21 (30.43%)	
	Swine	135 (31.18%)	8 (11.59%)	
Number of years in operation	6-30 years	270 (62.4%)	50 (72.5%)	0.137
	1-5 years	163 (37.6%)	19 (27.5%)	

[a.] USDA Zone information can be found online: https://planthardiness.ars.usda.gov Three USDA zones were used to categorize participating farms: zone 7b (-15 to -12.2 ºC), 9a (-6.7 to -3.9 ºC) and 9b (-3.9 to -1.1 ºC).
[b.] Organic: Some farms use organic practices but were "not certified" organic by a third party.
** No missing values; two surveys not completed.*

Final multivariable model results

The final mixed effect multivariable logistic regression model is shown in Table 1.6 and was chosen based on the lowest AIC and smallest deviance, relative to other models. The AIC of the final model was 369.5, with a deviance of 351.5. The highest variance inflation factor for any of the variables in the model was only 2.02, which was below our threshold of 5. The number of years a farm operated was identified as a confounder and adjusted in the final model.

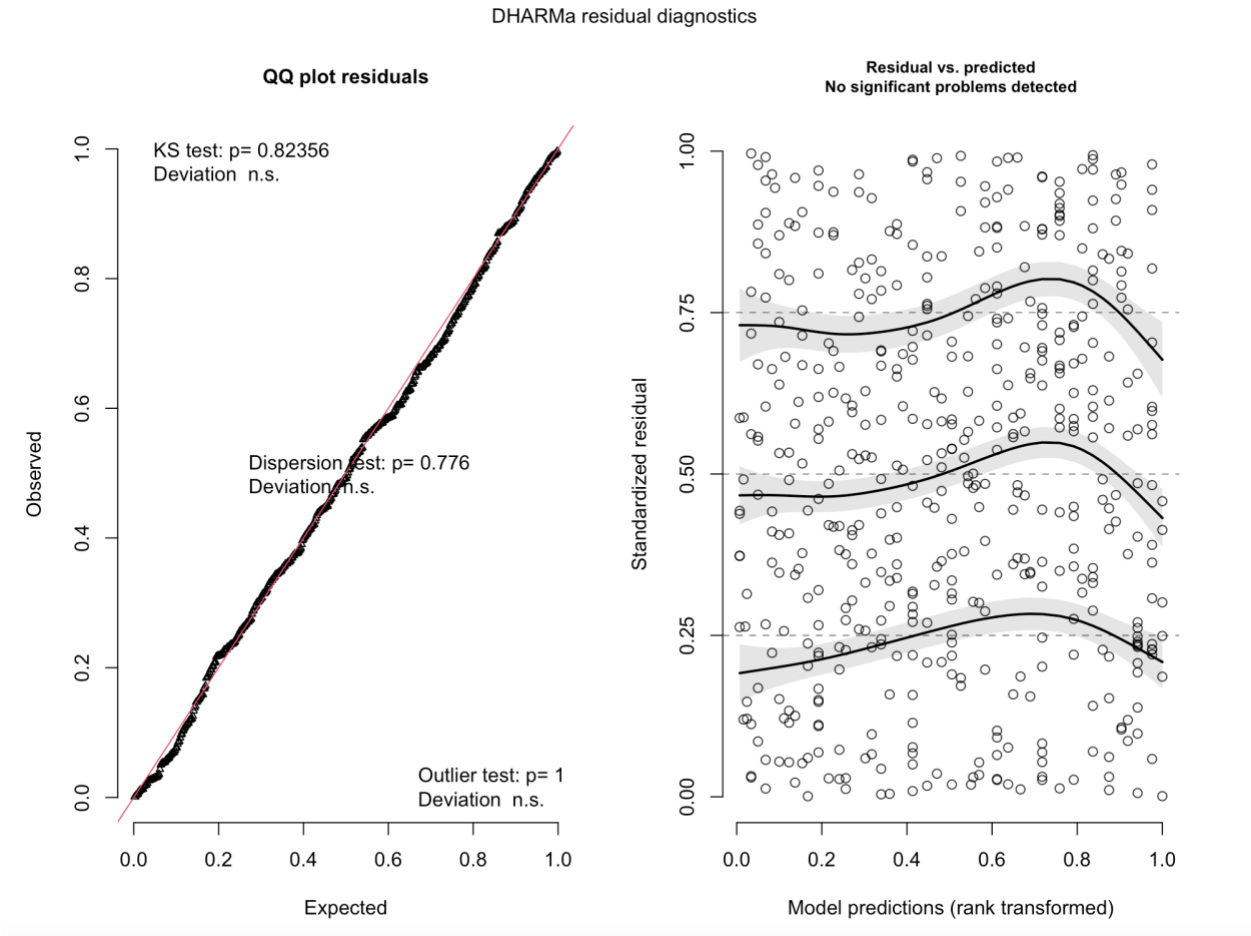
Table 1.6: Association between risk factors and the presence of Shiga toxin-producing *Escherichia coli* in fecal samples (n=502) collected from 14 diversified small-scale farms in California between May 2015 and June 2016, as demonstrated by a generalized linear mixed model, with farm as a random effect.

Variable	Level	Estimate	OR	95% CI	p-value
Intercept		-2.57			0.001*
Daily maximum temperature ºC	*numeric*	-0.06	0.95	0.91 - 0.98	0.003*
Sample source species	*Swine*	*Reference*			
	Goats	0.97	2.64	0.90 – 7.70	0.076
	Sheep	1.67	5.29	1.80 – 15.51	0.002*
	Cattle	1.53	4.61	1.64 – 12.96	0.004*
Multiple species shared a barn	*No*	*Reference*			
	Yes	1.83	6.23	1.84 – 21.15	0.003*
Livestock were allowed contact with wild areas	*No*	*Reference*			
	Yes	1.29	3.63	1.37 – 9.62	0.009*
Number of years in operation	*6-30 years*	*Reference*			
	1- 5 years	-0.98	0.38	0.13 - 1.11	0.076

** Indicates statistical significant variables with a p-value < 0.05*

Diagnostically, the simulated residuals versus predicted values did not show any significant problems: the Q-Q plot of DHARMA simulated scaled residuals was linear with no major deviations, and the Kolmogorov-Smirnov test indicated no deviation from uniform distribution of the scaled residuals (see Figure 1.2). The adjusted ICC was 0.08 for the final model, which signifies the proportion of the variance that is explained by farm clustering and indicated the need for a farm random effect. The isSingular test function in the lme4 package was false, which indicated no singularities existed in the final model.

Figure 1.2: Q-Q plot of simulated scaled residuals (left) and residuals versus predicted values plot (right) from the DHARMa package in R.



Interpreting significant parameters in the final model (i.e., confidence interval does not contain 1.0) while holding all others constant on average: for every ºC increase in the daily maximum temperature, the odds of a STEC positive sample decreases (OR = 0.95; $CI_{95\%}$: 0.91-0.98). The odds of a positive STEC sample were 4.61 times higher for cattle (OR = 4.61; $CI_{95\%}$: 1.64-12.96) compared to swine and more than five times greater in sheep (OR = 5.29; $CI_{95\%}$: 1.80-15.51). Goats had two times greater odds of a positive STEC sample; however, this estimate was not significant (OR = 2.64; $CI_{95\%}$: 0.90-7.70). The odds of STEC increased by 6.23 times for those farms that house multiple livestock species within the same barn versus those farms that housed livestock separately (OR = 6.23; $CI_{95\%}$: 1.84-21.15). The odds of a positive STEC sample were more than three times greater (OR = 3.63; $CI_{95\%}$: 1.37-9.62) for a farm that allowed its livestock contact with wild areas (e.g., forest, wetlands) in comparison to a farm that did not allow its livestock to have contact with wild areas. The effect of the number of years a farm had been in 0peration was not significant (p-value 0.076; $CI_{95\%}$: 0.13-1.11), but was included in the model as a possible confounder.

Discussion

This is one of the first studies to describe the unique characteristics of diversified small-scale farms in California, while ascertaining significant associations between risk factors and the prevalence of STEC. This study detected STEC on more than half of the enrolled farms and in all the livestock species sampled. Moreover, O-serogroups isolated in this study included ones that cause serve illness in humans, including O157:H7, O26, O103 and O111.(79) Significant risk factors associated with the presence of STEC included the daily maximum temperature, whether multiple livestock species shared a barn, the livestock source of the collected fecal sample, and whether livestock had contact with wild areas.

Overall STEC prevalence measured for the 16 farms in this study was 13.62%. Six of the 16 farms had 0% STEC prevalence; however, due to the intermittent shedding of STEC which may be affected by many factors, this result does not necessarily indicate that they are free from STEC. Although STEC prevalence in livestock raised on large farms has been measured frequently in past studies, evaluation of STEC prevalence and associated risk factors estimated on DSSF is less common. (74)(96)(97)(19) However, a study conducted by USDA-APHIS collected fecal samples from dairy cows in 21 states and stratified *E. coli* O157:H7 prevalence between large dairies (i.e., 500 or more cows) and small dairies (i.e., 100 cows or less) and reported that small ranches had 29.4% *E. coli* O157:H7 and large dairies had 53.9% prevalence.(98) Although this USDA-APHIS study indicated that small farms have less *E. coli* O157:H7 than large farms, the 29.4% prevalence they detected on small dairies is still larger than the 18.18% we identified in dairy cattle. Risk factors for the transmission of foodborne pathogens on large farms may be different, especially if they only raise one crop or livestock type, instead of a diversity of species.

One of our studies published in 2018 measured a 4.17% STEC prevalence in sheep raised on a mixed crop-livestock organic farm in California, which was lower than the 13.4% prevalence in sheep identified in this current study.(20) A study that screened livestock at the California state fair in 2005, which usually hosts livestock raised on small farms or in backyards, observed a 3% prevalence of *E. coli* O157:H7 in pigs, but did not find O157:H7 in any other livestock samples including dairy cows, whereas our study identified O157:H7 in cattle but not pigs.(99) A 2002 study that also collected fecal samples at fairs in three states, identified an *E. coli* O157:H7 prevalence of 11.4% in cattle, 1.2% in swine and 3.6% in sheep and goats, whereas we measured a 5.31% (6/113) *E. coli* O157:H7 in all cattle (i.e., combined dairy and

beef cattle samples).(100) Differing STEC prevalence in these aforementioned studies may reflect different management practices on farms or other climate or animal-level factors. Additionally, since ruminants are the main reservoirs for STEC, our results indicating that STEC prevalence in swine is lower comparatively than the other sampled species (i.e., cattle, goats and sheep) is in agreement with previous research, however, pigs are still a livestock species of public health concern, as they harbor *E. coli* O157:H7 as indicated by many studies.(100)(96)(101) Our model results also indicated that cattle and sheep are a significant factor in STEC presence on farms, as compared to goats and pigs. However, differences in location, laboratory methods and sampling methods make comparison between studies challenging.

More than half of the identified O-serogroups in this study are on CDC's list of the top 7 STEC of concern for public health, including six O157:H7, twenty-two O26, nine O103 and one O111.(79) *Stx2*, which is the more virulent form of the Shiga toxin gene that has been implicated in severe human disease, was identified in 16.46% (13/79) of O-serogroups; 13.92% (11/79) contained both *stx1* and *stx2*. The *eaeA* gene, which allows STEC bacteria to attach to human host cells, was detected in 55.69% (44/79) of positive STEC samples, contrary to a study conducted by Dewbury *et al*, which rarely discovered *eaeA* in their non-O157 isolates from cattle fecal samples.(102)(103)(104)(105) The *ehxA* gene, which is reported in severe human cases of STEC, was detected in 88.61% of the positive isolates (70/79).(104) Compared to a study conducted by Djordjevic *et al* in adult sheep and lambs, they detected *stx1*, *stx2* and *ehxA* in 78.2% of their positive serogroups, versus our study which only identified those three genes in 1.27% (1/79) of positive serogroups. However, they reported 0.8% of their serogroups had just *stx2* and *ehxA* genes, whereas in this current study, 11.39% (9/79) of the positive isolates

contained these two virulence genes.(104) The pathogenic STEC O-serogroups, genes and virulence factors identified in this study highlight the need for continued studies on DSSF, as well as outreach to stakeholders regarding pre-harvest food safety risks and development of on-farm mitigation strategies.

Significant risk factors identified by the final mixed effect model included daily maximum temperature °C. The data in our study ranged from 11.7°C – 39.80°C. An experiment that measured the decline of *E. coli* O157:H7 in inoculated manure at four temperatures, 7°C, 16°C, 23°C and 33°C, reported that *E. coli* O157:H7 declined significantly faster in manure at 23°C and 33°C, than at 7°C and 16°C, for both oscillating and constant temperatures.(106) This study confirms our model result, which suggested that as the daily maximum temperature increased, the odds of finding STEC in a fecal sample was less likely. A study by Franklin *et al* (2013) also identified daily maximum temperature as a significant risk factor, when conducting a study of STEC in wild ungulates in Colorado.(107) They detected a positive association between temperature and STEC presence in fecal samples, whereas our model identified a negative association with the daily maximum temperature.(107) However, the range of daily maximum temperatures displayed in their analysis were narrower than our recorded daily maximum temperatures, which may account for this difference.(107) Although many studies indicate that STEC sheds more in summer months, California microclimates differ from each other and from the majority of seasons in other states.(89) California valleys and foothills experience low humidity and temperatures above 37.78°C in the summer and autumn, which may affect STEC shedding from livestock raised on California farms located in different microclimates.(98) For instance, to compensate for the numerous microclimates in California in our study on *Campylobacter* spp., which included the same farms included in this current STEC study, we

divided the California summer season into Coastal and Inland and season was a significant risk factor in that final multilevel logistic regression model.(14) Interestingly, our *Campylobacter* study also found a significant association between presence of *Campylobacter* spp. and a farm owning swine, with 13.76% prevalence of *Campylobacter* spp. measured in pigs raised outdoors.

Difference in climate conditions (i.e., humidity, temperature range) between states in the US reveal a need to report the full range of temperatures and other environmental factors measured for studies estimating the effect of weather on foodborne pathogen shedding in livestock. For instance, a study that collected samples from conventional dairy and beef cattle in Michigan revealed that high average temperatures (more than 28.9°C) measured one to five days before sampling had a 2.5 times greater odds of STEC than lower temperatures, which differs from our study results that suggested that STEC survival is less likely at higher maximum temperatures.(22) Michigan results contradict ours, however the highest maximum daily temperature measured in our study (i.e., 39.8°C) is not a temperature normally observed in many areas of the US. The range of daily maximum temperatures for the Michigan study was 22.78 – 32.2°C, with one 36.11°C outlier. Additionally, our study included winter temperatures, while their study was only conducted in summer (i.e., May through August for both 2011 and 2012). Extreme temperature, heat index or humidity values observed in different parts of the world may affect conclusions and interpretations of results, especially between studies.

Stanford *et al* (2017) reported the effects of severe weather events on STEC shedding in Canadian cattle. (108) Although they also observed that STEC prevalence increased when ambient temperatures were higher than 28.9°C, a separate finding indicated that the O-serogroup O26 had a significant (p-value < 0.05) reduction in prevalence during extreme heat in July and August.(108) Almost 28% of the O-serogroups in our study were O26, and the final model

results may have been influenced by this strain. The ways that different non-O157 STEC strains react to varying environmental conditions, such as temperature or humidity, may account for variations in results between studies.(108) Moreover, changes in the host species during various temperature fluctuations or extreme weather events should also be studied.(109) For instance, Dawson *et al* (2018) measured behavioral changes in cattle during increased temperatures, as a possible driver of changes in STEC prevalence, such as increased water consumption or change in grazing habits. (109) Their simulation results indicated that higher summer temperatures may encourage more resting by cattle in crowded areas, such as under shade trees, which can lead to direct transmission of STEC.(109) Since the aforementioned studies differ in conclusions regarding the direction of environmental effects on STEC shedding in livestock, this risk factor needs further investigation, as perhaps there are underlying mechanisms accounting for the difference between results, including microclimates or animal level factors.(108)(75)

Our multivariable model also indicated that livestock sharing a barn with other animals resulted in 3.5 greater odds of a positive STEC sample. Multiple livestock housed in a barn could share pathogens by cross-contamination of food or water troughs or persistence of STEC in a barn environment that may not be subjected to regular cleaning. (77)(110)(111) Other studies have indicated that STEC persists for long periods of time in barns or on surfaces within the farm environment. For instance, one study swabbed multiple barn surfaces at a dairy ranch and measured 14.9% - 19.1% STEC in samples from cattle or calf feeders, and 11.3% – 18% on other surfaces.(112) Another study implicated water troughs as harboring *E. coli* O157:H7, and inferred that shared water troughs play a key role in the persistence and maintenance of continued *E. coli* O157:H7 infections in cattle.(113) A British study reported that housed beef cattle shed more STEC than unhoused and suggested that this may be due to shared water

sources or feeding bins and an accumulation of pathogens in a shared environment.(110)

Finally, the last significant risk factor from the multivariable final model indicated that livestock in contact with wild areas, such as forests or wetlands, have a higher likelihood of STEC presence in their feces. Wildlife, including feral pigs, deer, rodents and birds are known reservoirs of STEC. (114)(115)(42)(32) A study conducted in California identified a low prevalence of *E. coli* O157:H7 in rodents (0.2%), however, they did not test for non-O157 STEC in samples, which may have a higher prevalence in rodents.(116) A 2016 published study discovered the *stx2* gene in over twenty percent of Canada geese fecal samples and seven percent of nearby water samples from Lake Eric bordering Ohio, USA.(115) A case-control study conducted after 15 human cases of *E. coli* O157:H7, identified the source of STEC as those who ate fresh strawberries contaminated by deer feces.(32) Livestock that graze in wild areas may be exposed to indirect sources of STEC, for instance through environmental contamination of soil or water, or because wildlife that live in these bordering wild areas enter agricultural areas and contaminate the pastures grazed by farm animals.(117)(82)

Limitations of this study include the small sample size of farms that were convenience sampled, so the model results are not generalizable to other regions and farms. Moreover, because we collected the freshest fecal samples available and did not randomize sample collection, we may have added bias to the study results. Unmeasured variables that should be included in future studies include the age of the animal and whether livestock have direct or indirect contact with neighboring livestock.

Conclusion

Many consumers perceive diversified small-scale farms and outdoor-raised livestock as safer and more humane than food grown on large-scale conventional farms or meat animals

raised in confinement systems. However, identification of STEC O-serogroups that are of public health concern indicate the need for mitigation strategies, such as housing livestock species separately and restricting access to wild areas, to keep food safe by evaluating the risk factors and management practices that lead to the transmission of foodborne pathogens in a pre-harvest environment.

Acknowledgements: The authors thank all the farmers and ranchers who confidentially participated in this study. Thank you also to staff at Western Center for Food Safety and Pires lab for fieldwork and technical support.

Funding: This project was supported by start-up funds provided by the School of Veterinary Medicine, the Division of Agriculture Natural Resources (UC ANR), University of California and by the National Institute of Food and Agriculture, U.S. Department of Agriculture, under Award No. 2011-51181-30767. Any opinions, findings, conclusions or recommendations expressed in this publication are those of the author(s) and do not necessarily reflect the view of the U.S. Department of Agriculture.

Chapter 2

Identification of high-risk contact areas between feral pigs and outdoor-raised pig operations in California: implications for disease transmission in the feral pig-domestic pig interface

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Abstract

Although a majority of commercial swine production in the United States (US) occurs indoors with high levels of biosecurity, the US is currently experiencing a return to raising domestic pigs outdoors, due to consumer demand for sustainably-raised animal products. A challenge in raising pigs outdoors is the possibility of these animals interacting with wildlife disease reservoirs, such as feral pigs, and the associated risk of pathogen transmission. California has one of the largest and widest geographic distributions of feral pigs. Locations at greatest risk for increased contact between both swine populations are those regions that contain feral pig suitable habitat located near outdoor-raised domestic pig premises.

The main aim of this study entailed spatially identifying potential high-risk areas of disease transmission between these two swine populations. This aim was achieved by a three-step process: 1) predicting suitable feral pig habitat in California using a species distribution modeling method, Maximum Entropy (MaxEnt); 2) mapping the spatial distribution of outdoor-raised pig operations (OPO) in California; and 3) identifying high-risk regions where there is spatial overlap between feral pig suitable habitat and OPOs.

MaxEnt produces a prediction map with estimates of the relative probability of suitable feral pig habitat within each pixel, using presence-only points and predictor rasters. For feral pig presence data, we obtained feral pig hunting tags recorded with GPS coordinates. Publicly available biotic and abiotic predictor layers were included in variable selection steps for model building. To create the risk map for California, the final MaxEnt model predicting suitable habitat for feral pigs was overlapped with the location of OPOs in California to categorize areas at greatest risk for disease transmission. Five variables were identified as important in predicting suitable feral pig habitat in the final model including the annual maximum green vegetation

fraction, elevation, the minimum temperature of the coldest month, precipitation of the wettest month and the coefficient of variation for seasonal precipitation. The risk map reflects areas at greatest risk for contact between feral swine and domestic pigs raised outdoors and subsequent potential disease transmission.

Since raising pigs outdoors is a remerging trend, feral pig numbers are increasing nationwide, and both groups are reservoirs for various pathogens, the contact between these two swine populations has important implications for disease transmission in the wildlife-livestock interface.

Introduction

Although a majority of commercial swine production in the United States (US) occurs indoors with high levels of biosecurity, the US is currently experiencing a return to raising domestic pigs outdoors.(38)(118) Before the 1950s, most swine operations in the US were small-scale family farms and either a hybrid of indoor/outdoor or solely outdoor-based.(39)(38) Beginning in the 1960s, commercial swine production began transitioning to indoor systems, based on goals to increase efficiency and reduce swine disease transmission (e.g., brucellosis) as well as a public health mandate to decrease human trichinosis cases.(40)(119-121) However, consumer demand for sustainable or pasture-raised animal products within the past few decades has revived traditional methods of raising swine outdoors or on pasture (i.e., outdoor-raised pigs, pasture-based).(38)(118)(122)(123) While primarily considered a niche production method in the US, outdoor-raised pig operations (OPO) (e.g., commercial pork producers, backyard operations) are broadly distributed throughout California.

A challenge in raising pigs outdoors is the possibility of these animals interacting with wildlife disease reservoirs, such as feral pigs, and the associated risk of zoonotic and/or swine pathogen transmission (43)(37)(56)(124-126)(62) Both domestic and feral pigs share the same genus and species (*Sus scrofa*) and can be reservoirs for zoonotic pathogens (e.g., swine influenza virus, Shiga toxin-producing *Escherichia coli*) (19)(127-129)(17)(58)(45) Also, swine diseases eradicated in conventional indoor-raised herds (e.g., pseudorabies, brucellosis) have been documented in feral swine in California and contact between feral pigs and outdoor-raised swine herds is a risk factor for the reintroduction of these diseases to domestic herds in the US. (120) (62)(128)(121)(130-132) For example, a 2016 human case of brucellosis in New York state was traced to a feral pig intrusion event on a pasture-raised pig farm. *Brucella suis* was then

transmitted to domestic pigs raised outdoors in 13 other states through animal sales.(131)(60) Feral pigs could also play a significant role in the transmission and maintenance of transboundary animal diseases (TAD) introduced to North America.(37) (133)·(57)(126) For instance, African Swine Fever (ASF) is actively spreading in eastern Europe, with wild boars transmitting this devastating disease between and within countries.(134) Similarly, wild boars abet the transmission of ASF in South Korea, spreading the virus to outdoor-raised swine.(135)(136) And most recently, ASF was identified in domestic swine in the Dominican Republic, which is the closest to the US that ASF as spread in this century.(55)

During the past few decades, feral pig populations have greatly increased in the US from 17 to 41 states.(137-139) California has one of the largest and widest geographic distributions of feral pigs and this invasive species has the broadest habitat range of any large mammal except humans, which is in part due to their ability to adapt to a diverse range of ecological habitats and their opportunistic omnivore diet.(34)(140)(49)(53)(51)(139) Feral pig population distribution and abundance is dynamic yet has not been documented at fine spatial units. Previous presence maps reported feral pigs for an entire county, even if there had only been a single occurrence recorded countywide.(140-142)

Hypothetically, an area is at higher risk of disease transmission if it is more likely to experience interactions between feral pig and domestic pigs raised outdoors, as these outdoor-based pigs can serve as a conduit for disease spread from wildlife to humans. Locations at greatest risk for increased contact between both swine populations are those regions that contain feral pig suitable habitat located near outdoor-raised domestic pig premises, especially those OPO with relatively low levels of biosecurity.(130)·(132)·(143)(139) Contact between feral pigs and outdoor-raised pigs in California has been documented, as feral pigs are attracted to

agricultural regions for food, water and mates.(57)(56)(59)(43)(144-146) There is enormous value in identifying agricultural regions with a higher probability of feral pig contact, because these areas could benefit from targeted cost-effective disease surveillance and risk-mitigation strategies to prevent disease transmission.

Predicting suitable habitat for feral pigs (i.e., likelihood of feral pig presence) in combination with spatially characterizing the distribution of OPO can provide an important tool to ascertain possible high-risk areas of contact at the feral-domestic pig interface and identify future disease spillover areas.(145)(147)(148) Species distribution modeling (SDM) methods have been widely used in ecological studies and are becoming popular for use in epidemiological investigations of disease transmission between wildlife and livestock.(149-152)(146) Maximum Entropy (MaxEnt), which is one type of SDM, allows usage of presence-only data for the species of interest (i.e., feral pigs).(153) In combination with biologically-appropriate covariate factors, MaxEnt is able to spatially predict the probability of suitable habitat for a species for a chosen spatial unit (i.e., pixel). (154)

These two parallel trends of expanding feral pig populations and a resurgence of raising domestic swine outside has important implications for disease transmission, which could negatively impact both public health and California's agricultural industry. To the best of our knowledge, there are no maps characterizing where suitable feral pig habitat overlaps with domestic pigs raised outdoors at the farm-level in California. The overall objective of this study entailed spatially identifying potential high-risk areas of disease transmission between these two swine populations. This objective was achieved by a three-step process: 1) predicting suitable feral pig habitat in California using MaxEnt; 2) mapping the spatial distribution of OPO in

California; and 3) identifying high-risk regions where there is spatial overlap between feral pig suitable habitat and OPOs, as potential disease transmission areas.

Materials and Methods

Maximum Entropy model

MaxEnt is an established SDM method that produces an output prediction map containing estimates of the relative probability of suitable habitat areas for the species of interest (i.e., feral pigs) within each pixel, using presence-only points and predictor rasters (i.e., covariate layers). (136)(155-160)(148) (153)(144) For feral pig presence data, we obtained feral pig hunting tags from 2012-19 that were cleaned and recorded with GPS coordinates by the California Department of Fish and Wildlife (CDFW). Hunters in California are voluntarily asked to report feral pig harvest locations by submitting hunting tags to CDFW. Using hunting records for presence-points of feral pigs or wild boars has been used in previous studies.(161)(146) CDFW 2012-19 feral pig hunting tags totaled 5,148 after removing duplicates. Due to the large amount of data points, hunting tags were also manually filtered (i.e., subsampled) by year as a way to decrease the abundance of points before running models to reduce sampling bias and increase model stability, as suggested by previous analyses of MaxEnt.(146)(160)(162-166)

Publicly available predictor layers online, including biotic (e.g., land cover, vegetation) and abiotic (e.g., temperature, precipitation, elevation), were included in variable selection steps, see Table 2.1. These predictors were chosen based on known feral pig behaviors, habitat and food preferences.(34)(144)(167-170)(52)(50) For instance, AVGMODIS was the annual maximum green vegetation fraction (MGVF) combined with 12 years of normalized difference vegetation index data (NDIV) and relates to food and shrub cover for feral pigs.(170-173) Other variables included elevation, as feral pigs may prefer specific altitudes, and nineteen

environmental variables from the WorldClim set of 30 year trend climatic factors.(174) Examples of environmental variables used from the WorldClim site included BIO6, which is the minimum temperature of the coldest month, BIO13 which represents precipitation of the wettest month and BIO15 which is the coefficient of variation for seasonal precipitation.(175)

Table 2.1: Publicly available predictor layers assessed during variable selection for Maximum Entropy model building.

Name	Description	Source
AVGMODIS*	Annual maximum green vegetation fraction, 12 years of normalized difference vegetation index data	modis.gsfc.nasa.gov/data/
CropScape	USDA National Agricultural Statistics Service Cropland Data Layer	https://nassgeodata.gmu.edu/CropScape/
ELEVATION*	Elevation/Altitude	www.worldclim.org/
FVEG	Raster representation of statewide vegetation with WHR types, WHR size and WHR density	https://frap.fire.ca.gov/mapping/gis-data/
GAP	USGS GAP analysis project: land cover	https://www.usgs.gov/core-science-systems/science-analytics-and-synthesis/gap/science/
Global Human Influence Index	Nine global data layers: human population pressure, human land use and infrastructure, and human access	https://sedac.ciesin.columbia.edu/data/set/wildareas-v2-human-influence-index-geographic/maps
NDVI	Normalized difference vegetative index	https://data.nodc.noaa.gov/cgi-bin/iso?id=gov.noaa.ncdc:C00813
NLCD	National Land Cover Database	https://www.mrlc.gov/data
Open Water	Multiple integrated global remote sensing-derived land-cover products and prevalence of 12 land-cover classes	http://www.earthenv.org/
PRISM	Seven climatic variables for the US. Annual and monthly precipitation temperature and other data	http://www.prism.oregonstate.edu/
Streams	Streams in the US, used to measure distance to water	https://catalog.data.gov/dataset/cdfg-100k-streams-2003
USDA zones	Hardiness zones based on mean extreme annual minimum temperature	https://planthardiness.ars.usda.gov/
WorldClim*	19 Bioclimatic variables: 30-year averages 1970-2000	www.worldclim.org/bioclim

** Indicates variables included in the final model.*

MaxEnt models were built in R Statistical Software version 0.98.110253 ©.(95) The following R packages were used to run MaxEnt: dismo, sp, and raster.(156)(176-179) MaxEnt settings were chosen based on previously published literature and included using 25 random test points, 15 replicates, 5000 maximum iterations and the 10-percentile training for the threshold rule. (149),(152)(156),(154) (180) A regularization multiplier of 1 through 5 was assessed to avoid overfitting and the default 1 was determined to be the optimal setting for the final model.(180) Logistic values for output was used as well as cross validation, which separates presence points into 80% training and 20% testing data (i.e., model validation), using k-fold sub-sampling to fit a model.(149)(154)(181) The relative contribution of each variable in a MaxEnt model was assessed comparing both percent contribution and permutation of importance, averaged over the number of iterations run and ascertained by jackknife tests.(149)(181)(153) Predictors for the final model were assessed using a backward variable selection approach: variables remained at each step if their percent contribution or permutation importance was approximately 10% or more.(136)(149) The response curves generated within MaxEnt showed the predicted probability of suitable feral pig habitat for each individual variable, changing per each level of the predictor.(50)(167)(182)

MaxEnt model performance was assessed using the area under the curve (AUC) of the receiver operator characteristic (ROC), averaged over the number of chosen replicate runs. (149)(152)(183) AUC reflects a model's prediction ability, on a scale of 0 to 1.00, with 0.50 representing random chance. While AUC is a standard diagnostic method to evaluate MaxEnt models, some authors suggest calibrating the AUC (i.e., AUCc), which removes spatial sorting bias (ssb) (i.e., spatial autocorrelation) by using point-wise distance sampling.(184)(156)(176) A ssb close to 1 indicates no spatial sorting bias, whereas a ssb close to 0 suggests a large spatial

bias, and the need to use AUCc.(184) The final model was chosen based on the highest AUCc, relative to other models.

Risk Map and OPO

The feral pig-domestic pig risk map was built by overlapping California OPO locations with the final MaxEnt feral pig suitable habitat raster. Between 2014-2019, a list of California OPOs was compiled through various sources (e.g., agricultural festivals, local farmers markets, University of California Cooperative Extension (UCCE) advisors, web-based searches (search terms: “pasture-raised pork”, “pastured pigs”). GPS coordinates for all OPO were identified using Google Earth Pro v7.3.3.(185) Additionally, an online survey that contained an interactive map component was built with Survey 123 v3.6.(186) The survey contained 29 questions that consisted mainly of multiple choice questions, with a few open ended questions about the number of animals raised (e.g., how many sows or boars raised on average each year). The survey included questions regarding biosecurity practices, swine health and feral pig presence. This online survey was announced electronically (e.g., media, e-newsletters) to swine related groups and organizations or conducted in-person at events, such as agricultural fairs. The survey instrument and protocols were reviewed and exempted by the Institutional Review Board (IRB) of the University of California-Davis (No. 1180798-1).

To build a risk map for California, the final MaxEnt model predicting suitable habitat for feral pigs was overlapped with the location of OPOs to categorize areas at greatest risk for disease transmission, due to contact between these two swine populations, and characterize risk at the farm-level. The underlying assumption presumed that direct or indirect contact between feral pigs and domestic pigs raised outdoors is a risk for disease transmission. The probability of suitable habitat for feral pigs was extracted from the final MaxEnt model for each OPO location,

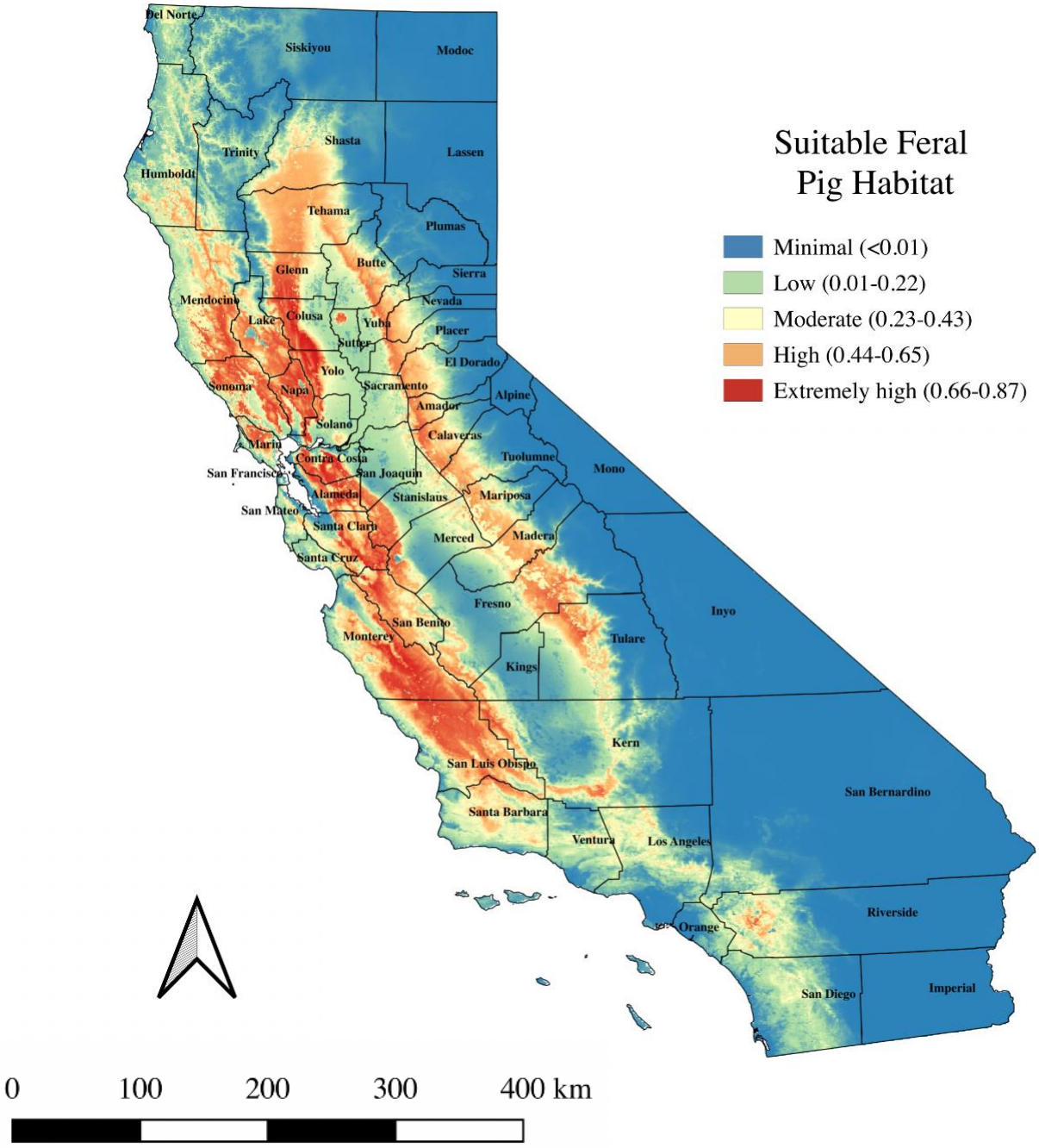
using the Sample Raster Value tool in QGIS and added to the OPO shapefile. Then the Kernel Density tool in QGIS was used to make the risk map, matching the 270m x 270m resolution of the MaxEnt model and using the MaxEnt model probabilities as weights. Additionally, we used a radius of 5 km at each OPO location, which was an extrapolated average estimate from US based studies that measured home range of feral pigs, understanding that home ranges vary depending on age and gender of animal, as well as resource availability.(187)(188)(144)(52) The Kernel Density map was overlaid with the final MaxEnt model.

Results

MaxEnt model results

The final MaxEnt model was chosen based on the highest AUCc of 89.7, relative to other models (see Figure 2.1). Probability values that predict suitable habitat were divided into five equal interval categories: minimal (< 0.01); low (0.01-0.22); moderate (0.23-0.43); high (0.44-0.65); and extremely high (0.66-0.87), with 0.87 being the highest predicted probability in the final MaxEnt model. Areas with the highest likelihood of suitable feral pig habitat in California (i.e., orange, and red categories) included the north coast from Mendocino County all the way south along the coast to Santa Barbara County, and counties that border these coastal counties (e.g., Lake, Napa, Contra Coast, Santa Clara and San Benito). Additionally, suitable habitat areas included the foothills of the Sierra mountains, from Shasta County south to Tulare County. Least likely suitable habitat included the Central Valley and eastern counties of California, from the most northern county of Modoc all the way to Imperial County in the south.

FIGURE 2.1 Final MaxEnt model predicting suitable feral pig habitat in California. Color-coded categories represent the probability of suitable feral pig habit on a scale of almost zero (<0.01) to extremely high (0.66-0.87), based on equal intervals.



Five variables were identified as significant in predicting suitable feral pig habitat in the final model based on 2017 hunting tags (n=1,745). The five significant variables were AVGMODIS, Elevation, BIO6, BIO13 and BIO15. All five variables provided approximately 10% or more percent contribution and permutation importance to the final model. (Figure 2.2). The jackknife test results provided more information regarding the importance of each variable in the final model (Figure 2.3). For example, BIO15 was the variable with the highest gain when used alone and elevation had the most information that was not available in the other variables. The response curves for the significant five variables indicated the predicted suitability range of each variable for feral pigs (i.e., the x-axis values above 0.50 on the y-axis). (Figure 2.4) For instance, feral pigs are predicted to prefer vegetative cover (i.e., AVGMODIS) of at least 60% or more.

Table 2.2: The analysis of variable contribution table provided estimates of the relative contribution of each variable to the final MaxEnt model.

Variable	Percent Contribution	Permutation Importance
BIO6: minimum temperature of the coldest month	28.2	21.4
AVGMODIS: Annual maximum green vegetation fraction	21.7	9.5
BIO13: rainfall of the wettest month	20.7	27.2
BIO15: Variation of annual rainfall	9.4	11.5
Elevation	20.1	30.5

Figure 2.2: Jackknife results for final MaxEnt model and indicates importance of key variables. BIO6 was the minimum temperature of the coldest month, AVGMODIS was the annual maximum green vegetation fraction, BIO13 was the precipitation of the wettest month, BIO15 was the variation of annual precipitation and elevation.

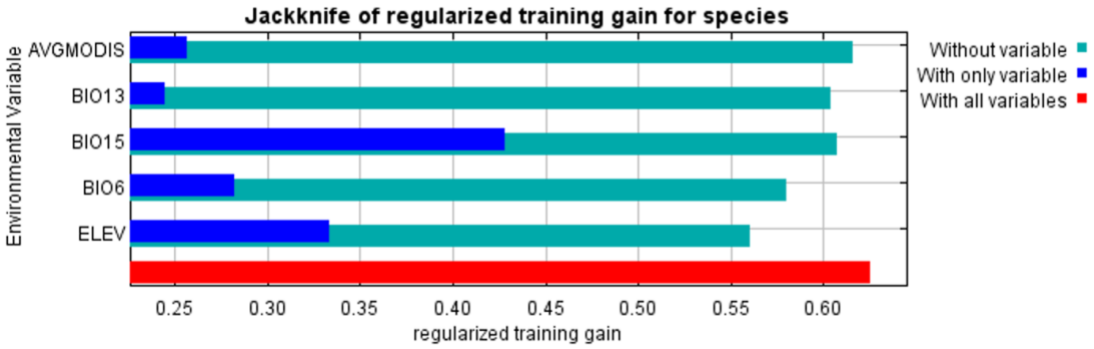
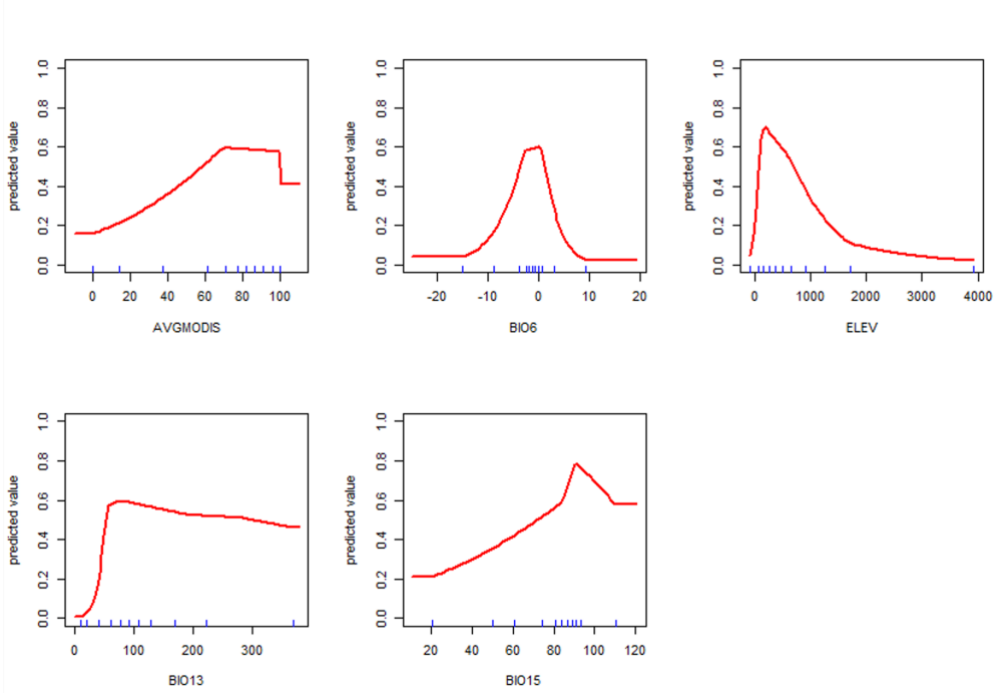


Figure 2.3: MaxEnt response curves for the five significant variables used in final MaxEnt model. The response curves generated with MaxEnt show the predicted probability of suitable feral pig habitat for each individual variable, for each level of the predictor. BIO6 was the minimum temperature of the coldest month, AVGMODIS was the annual maximum green vegetation fraction, BIO13 was the precipitation of the wettest month, BIO15 was the variation of annual precipitation and elevation.



Risk map and OPO

A total of 305 OPOs were identified between 2014-2019, from 79.30% (46/58) of California's 58 counties (i.e., no OPO data for 12 counties). The most OPOs were identified in the following counties: Sonoma (n=48), Mendocino (n=19), Nevada (n=16) and Yolo (n=12). From the online survey, 39 OPO locations were gathered from 44 respondents and included in the final total. All survey respondents raised domestic swine outdoors and 25.00% (11/44) had seen feral pig presence within two miles or less of their domestic swine raised outdoors, with 15.91% (7/44) observing feral pigs within 500 feet of their pig herd. Domestic pig herd size ranged from 1- 350 animals, with a mean of 24 and median of six. Acres dedicated to raising pigs ranged from $^{1}/_{16}$ to 30 acres with a mean of 4.67 and median of two acres, with nine not answering.

The risk map reflects areas at greatest risk for contact between feral swine and domestic pigs raised outdoor and subsequent potential disease transmission (Figure 2.5). Risk levels start at blue for low-risk areas and range up to orange and red for the highest risk areas. Areas with the most risk for contact between these two swine populations are denoted in orange or red, with sharper colors representing denser clustering of OPO. The counties with the highest likelihood of suitable feral pig habitat and densest clustering of OPO included: Sonoma, Marin, Napa, Yolo, Nevada, Mendocino and Lake counties. Areas at lowest risk include the full eastern edge of California, which includes the Cascadian and Sierra Nevada Mountain ranges as well as deserts in the south. Table 2 categorizes the distribution of OPO at each level of probable suitable feral pig habitat using the final MaxEnt model levels. The results indicated that 49.18% of OPO are located near extremely high or highly suitable feral pig habitat.

Figure 2.4: Risk map demonstrating areas in California at greatest risk for contact between feral pigs and outdoor-raised domestic pigs within a 5km radius from each farm, using the Kernel Density tool in QGIS. Colors are based on the probability of suitable feral pig habitat from the final MaxEnt model at each OPO, with sharper colors representing denser clustering of OPO. Included is an inset map of the Bay Area (see right figure).

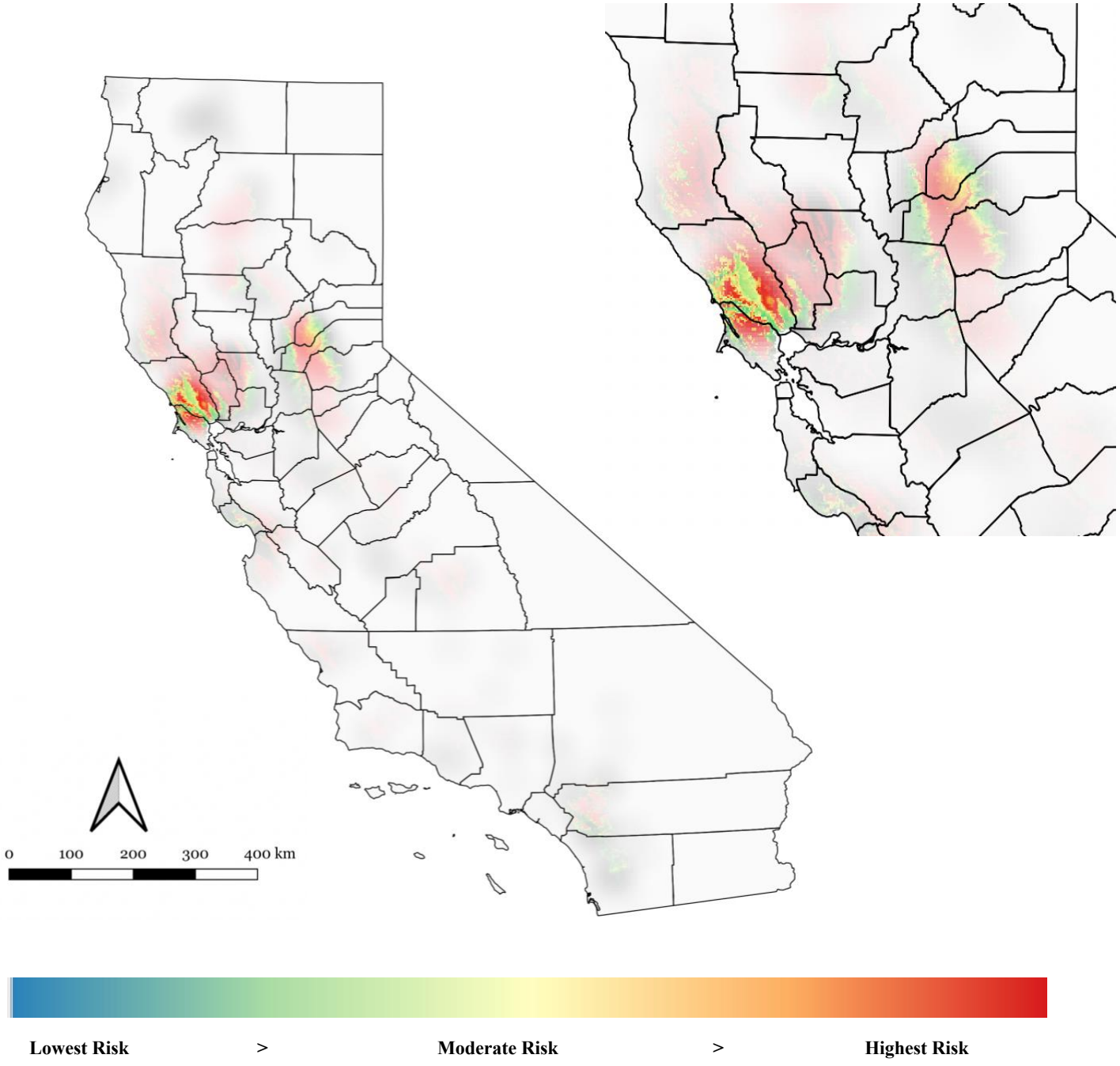


Table 2.3: Percentage of 305 OPO identified in each MaxEnt suitable feral pig habitat level. The final MaxEnt model contains a probability scale of 0.00 to 0.87 and was divided into equal intervals. Regarding the percentage of OPO located near suitable feral pig habitat, 49.18% of 305 OPO were identified in high or extremely likely suitable areas.

Levels	%OPO (ct/305)
Minimal (< 0.01)	0.98% (3/305)
Low (0.01-0.22)	19.67% (60/305)
Moderate (0.23-0.43)	30.16% (92/305)
High (0.44-0.65)	25.90% (79/305)
Extremely high (0.65 +)	23.28% (71/305)

Discussion

In this study, we built a feral pig suitable habitat prediction model for California using MaxEnt at a fine scale of 270m x270m. Significant predictors of suitable feral habitat included precipitation, minimum temperature, elevation, and percentage of vegetation. Additionally, this study overlapped MaxEnt predicted suitable feral pig habitat and outdoor-raised pig operations to create a risk map for potential disease transmission in California at the feral pig-domestic pig interface. To the best of our knowledge, this is one of the first studies that identified areas at risk for feral and domestic pig contact in California. Although previous studies discussed the possibility of feral pig populations spreading disease to outdoor-raised pigs at the county level, to our knowledge, this is the first study to predict risk at the farm-level in California.

Since the exact location of most feral pig populations is unknown, species distribution predictive methods like MaxEnt are important to understand where feral pigs could potentially

interface with domestic swine raised outside, either currently or in the future. Our final MaxEnt prediction model provides a more informative picture of suitable habitat for feral pigs than previous studies, which only showed single presence points or reported feral pigs at the county level, even if only one feral pig was identified in that county.(139-142) For instance, although previous county-level maps indicated that all California counties except for Imperial County harbored feral pigs, our MaxEnt model shows almost no suitable habitat in an additional five counties: Modoc, Mono, Alpine, Lassen and Inyo. This result may indicate that few feral pigs have been seen in those counties.

Additionally, the final MaxEnt model was based on a fine spatial scale and indicated heterogenous suitable habitat, not a uniform distribution, for each county, which is compatible with the fact that feral pigs need shrub cover and food to survive, which would not be found in cities or deserts.(51) Earlier feral pig mapping studies by the Southeastern Cooperative Wildlife Disease Study and National Feral Swine Program (NFSP) focused on the entire US and only county level occurrence of feral pigs.(141)(142)(189) A 2015 USDA study overlapped NFSP county-based feral pig locations with data from the 2012 NAHMS study of small-enterprise swine operations, specifically whether these survey respondents had seen feral pigs on their premises or within the same county, to ascertain the level of agreement between the two datasets.(139) They identified five counties in California that were in agreement with our MaxEnt model findings for suitable feral pig habitat: Mendocino, Tehama, Nevada, El Dorado, and San Luis Obispo, and two counties that differed: Ventura and Los Angeles counties. However, their map does not reflect the heterogeneity of feral pig habitat in each county nor identify high-risk contact areas between farms and feral pigs in California, as they did not identify any outdoor-raised domestic swine in California. Although these county-based maps are important to demonstrate the trend of increasing

feral pig populations nationwide, stakeholders and feral pig disease surveillance agencies could benefit from targeting outreach and mitigation strategies to specific regions within a county using our maps.

The results of our final MaxEnt model indicated five variables that were useful in predicting suitable feral pig areas in California, including three WorldClim layers: BIO6: the minimum temperature of the coldest month, BIO13: precipitation of the wettest month, and BIO15: the coefficient of variation for seasonal precipitation. Other studies also used WorldClim factors to predict the distribution of wild boar or feral pigs. These bioclimatic variables have been widely used in environmental studies and are now becoming popular for use in epidemiological investigations.(175) These climate variables are 30 year averages and "capture broader biological trends better than the temperature or the amount of precipitation for a given day due to the inherent variability associated with weather."(175) Bosch *et al* (2014) built a MaxEnt model for wild boar in Spain and their model also contained BIO6 and BIO15 as did regional models built by Pittiglio *et al* (2018) with BIO13 being significant as well. (170)(190) BIO6 is the minimum temperature of the coldest month and is interpreted as being a useful variable when deciding if the species of interest is affected by extreme cold events throughout a year.(175) Hill *et al* (2014) used MaxEnt to predict the distribution of *Trichinella* and *Toxoplasma gondii* in feral pigs in the US and also identified BIO6 and elevation as significant predictor variables, along with land cover and other WorldClim factors. (132) The response curve for BIO6 in our model peaks at the predicted ideal range for feral pigs, with both ends indicating extreme cold temperatures that may be avoided by feral pigs. A 2015 study by McClure *et al* (2015) indicated that suitable feral pig habitat may be limited by cold temperatures, precipitation and water availability, which reflects our findings.(144) BIO13 is defined as precipitation of the wettest month and is useful if extreme rainfall patterns

influence the range of feral pigs.(175) BIO15 measures the variation in annual precipitation totals per month (i.e., seasonality of precipitation) and reflects the variability of rainfall that may affect a species.(175) According to the Jackknife graph, the variable with the highest gain when used alone was BIO15, and therefore had the most important information for predicting suitable feral pig habitat. Snow *et al* (2017) used Bayesian methods to predict the expansion of feral pigs in the US , but also detected that temperature and precipitation levels were significant predictors.(140)

The final MaxEnt model gain is decreased the most if elevation is ignored and therefore it has significant information that is not available from the other variables in predicting feral pig suitability. Elevation was also significant in the MaxEnt models built by Hill *et al* (2014).(132) These results combined with the response curve possibly reflect feral pigs preference for lower altitudes in the US. AVGMODIS, a measure of the annual maximum green vegetation fraction on a scale of 0 to 100, was also an important predictor of suitability, which reflects feral pigs' need for available food and vegetative cover.(172) Garza *et al* (2018) identified NDVI, which AVGMODIS is based upon, and precipitation as important variables in predicting home ranges of feral pigs or wild boar worldwide, using generalized linear models.(167)

The significant layers identified in our study to predict feral pig suitability are not unique, and this may be due to the fact that feral pigs are a highly adaptable and opportunistic omnivores. (140) Lobe *et al* (2008) stated that MaxEnt AUC values will be lower for generalist species that are widely distributed.(191) However, the AUCc of our final model was 89.7, which indicates a good model. Additionally, 2017 hunting tags (n=1,745) vs. all 5,148 points for 2012-19 provided the best model. MaxEnt is an important method to predict the distribution of rare species, and an upper maximum range for the number of species occurrence points has not been previously determined. However, our result fits with a study conducted by Chen *et al* (2012) to determine the

sample size for the outcome variable in building MaxEnt models. They reported that standard deviation decreased and MaxEnt models became more stable using species occurrence points of 1,000-1,200.(162)(163)(166) Most likely the sample size of the outcome variable that reaches asymptote is dependent on geographic extent and characteristics of the species of interest.

Regarding feral pig presence on farms, the most recent NAHMS survey asked participating swine small-enterprise producers in the US (i.e., those raising 100 pigs or less) about presence of feral swine in their county but did not separate farms based on whether they raised domestic swine indoors or outdoors. However, a 2015 United States Department of Agriculture (USDA) report regarding overlap of feral and domestic pigs in the US used this NAHMS dataset and reported that of 320 participating US counties, 74% of these counties had small-enterprise swine producers who allowed their pigs some level of outdoor access.(139) The NAMHS results indicated that 52.9% of small-enterprise swine producers in the West/South region, which included California, reported feral pigs in the same county, with 16.2% of those having feral pig presence on their operation, similar to our survey results that showed 15.91% of respondents had seen feral pigs within 500 feet of their pig herd.(41) Another study that measured co-occurrence of feral pigs and agriculture to understand the risk of disease transmission, but did not separate outdoor versus indoor herds, reported that on average, 47.7% of all types of farms had feral pigs in the same counties, including California, showing a significant increase in the decade from 2002-2012.(54) The results of these aforementioned survey-based studies indicated together that more than 45% of farms have feral presence within the same county, which matches the results from our risk map that showed almost half of the identified OPO had suitable feral pig habitat nearby(139)(41)(54) These findings indicate the

need for targeted outreach and mitigation strategies for those farms at highest-risk for feral pig contact, due to the potential for disease transmission between these two swine groups.

Studies that identified high-risk areas in California between feral pigs and domestic swine raised outdoors are sparse. A 2015 USDA report extracted outdoor operations with NFSP feral swine populations and did not identify any hot spots of overlap in California as seen in our results. However, they did not report the number of OPO per state or county and most likely our state-focused study identified more OPO than their survey-based national study. A 2017 study by Miller *et al* also assessed possible disease transmission between feral pigs and farm at the county level.(54) They reported that domestic swine, either raised indoors or outside, have been increasing in counties that also had feral pig presence. The lack of maps identifying areas at high-risk for disease transmission between these two swine populations indicates a need for further research. Additionally, the risk map identified eastern counties as having the lowest risk. However, we did not identify OPO in many of these counties, therefore we cannot say there is no risk in these regions.

A limitation of this study involves using hunting tags as a proxy for presence of feral pigs to predict suitable habitat. Hunting tags are voluntarily submitted to CDFW by hunters and estimated to account for only 30% of all hunted pigs and most likely biased toward easy to access areas. Also, only half of the land in California is public land and accessible to hunters, therefore feral pigs hunted on private land are not included in our data sets. However, Rutten *et al* (2019) used similar hunting bags and MaxEnt to successfully predict the distribution of wild boar in Belgium.(146) And Alexander *et al* (2016) also used hunting records to predict wild boar habitat in Europe.(161) Additionally, MaxEnt assists in overcoming these challenges by identifying similar habitats in all parts of California and predicting suitable areas.

Both the MaxEnt model and risk map are limited because they are static maps that use fixed layers as their foundation; consequently, they do not incorporate dynamic events over various years (e.g., wildfires, landscape changes, weather fluctuations). Also, feral pigs may migrate seasonally due to shifting weather, resource availability, hunting pressure or wildfire and future research could focus on species distribution modeling that includes dynamic real time variables or remote sensing data; however, seasonal or dynamic spatial data are not available yet for most spatial predictors in California. (144)(146)(167)(187)(192-194) However, our approach is valuable as a first step in identifying multiple high-risk areas for future research, where additional data could be collected. Furthermore, future research could add feral pig disease data collected statewide to evaluate if high-risk areas for feral-domestic pig contact equates to those areas with higher prevalence of diseases.(147)

There are some challenges and limitations to the risk map generated in this study. For instance, farms and ranches in California, including backyard and commercial operations, are not required to register with state agricultural agencies, therefore, the total number, distribution, and size of OPOs remains unknown and are underrepresented in this study. A majority of the identified OPO in this study were commercial pork producers with an online presence or ones that attend conferences, farmers markets and fairs. If more OPO locations could be identified, than a more comprehensive map of high-risk areas could be generated. Additionally, because we are based at the University of California, Davis in Yolo County, there is selection bias in the OPO identified as our agricultural networks are within the UCCE network. Overrepresented counties reflected either sampling bias or clustering of these niche operations or both. Nevertheless, the number of OPOs included in this study (n=305) and the fact that more than 40% of these operations were in highly suitable areas for feral pig contact is relevant as an initial

approximation of a likely much larger risk of disease transmission at the feral-domestic swine interface in California. In the future, adding disease cases to this risk map would add additional epidemiological information regarding possible pathogen transmission.

Conclusion

This study evaluated the feral-domestic pig interface of two parallel trends: expanding feral pig populations and an increase in outdoor-raised pig operations in California, as related to the risk for future disease transmission. Since both swine populations are reservoirs for various pathogens, the contact between these two swine groups has important implications for disease transmission in the wildlife-livestock interface. This study provides a foundation to design targeted, cost-effective disease surveillance and risk mitigation programs in regions at highest risk for wild- domestic pig contact and can serve as a template for similar efforts nationwide. Moreover, the results of this study provide a framework to create an outreach extension program and inform all stakeholders (e.g., farmers, government agencies) that may be called upon to respond to future zoonotic or TAD outbreaks, such as ASF. The results of this study, despite limitations, can provide important information to stakeholders and organizations that handle swine diseases or public health problems originating from any swine group in California.

Funding: This study was supported by the Agriculture and Food Research Initiative grant no. 2019-67011-29609 / project accession no. 1019249 from the USDA National Institute of Food and Agriculture and a University of California, Davis Academic Federation grant.

Acknowledgements: The authors would like to thank the following people and organizations for their support and assistance: Robert Hijmans for Maximum Entropy expertise; Michele Tobias at UC Davis for QGIS assistance; Robert Johnson and Shane Feirer at UC ANR for building the online survey; Kristi Cripe and Craig Stowers at CDFW for hunting tag data and feral pigs information; Jim Thorne and Michele Stern for PRISM data, and big thanks to our feral pig experts: Dr. Dana Nelson, Dr. Rebecca Milhalco, Dr. Hector Webster, John Harper, and Dr. Roger Baldwin.

Chapter 3

Risk factors for Shiga toxin-producing *Escherichia coli* in domestic swine raised outdoors near feral pigs in California

Authors

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Abstract

The trend to raise domestic swine outside in the United States, instead of inside confinement barns, has been increasing in the last few decades and is a risk factor for the transmission of disease. Both feral and domestic swine are considered a species of concern for sharing of pathogens (e.g., Shiga toxin-producing *Escherichia coli (*STEC)). Outdoor-raised pig operations (OPO) are primarily considered a niche market; however, they are broadly distributed throughout California, providing an opportunity for the transmission of newly emerging or reemerging diseases.

The objectives of this study focused on a) measuring the prevalence of STEC in outdoor-raised domestic pigs and feral pigs located near these OPO, and b) analyzing risk factors for the presence of STEC on OPO that operate near feral pig populations in high-risk counties.

Fecal samples from outdoor-raised domestic pigs and feral pigs were collected. Sample collection was targeted to high-risk counties that had a higher likelihood of feral pig to outdoor-raised pig contact. STEC prevalence was estimated at the farm, type of pig, and county-level. Data analysis was conducted using generalized linear mixed models to identify significant risk factors for the presence of STEC in outdoor-raised pigs.

At the county level, STEC prevalence for feral pigs ranged from 0% to 25.00% and 0% to 57.14% for domestic outdoor-raised pigs. Overall, 35 out of the total 192 samples (18.23%) were positive for STEC, with five samples collected from feral pigs (11.63%; 5/43) (CI_{95} = 5.07% - 24.48%) and 30 samples gathered from domestic pigs (20.13%; 30/149) (CI_{95} = 14.48% - 27.29%). Three of the top seven serogroups that cause the most severe human illness in the US were identified in this study: O157:H7, O26 and O103.

Five of the 11 OPO (45.45%) had seen evidence of feral pig presence on their farm, with four (36.36%) stating that feral pigs had direct contact with their domestic pigs. Significant variables identified in the final multivariable logistic regression model were the juvenile age group of the sample source (OR = 8.66 ; CI_{95}: 2.11-47.89), distance to the nearest surface water, (OR = 0.03; CI_{95}: 0.00-0.23), and if the farm raised pigs in an area that had access to a wild area (OR = 18.24; CI_{95}: 2.94-520.31).

As the number of OPO continues to grow, evaluating risk factors that are unique to these operations will help identify mitigation strategies to keep food safe from farm to fork and protect California's agricultural economy.

Introduction

The trend to raise domestic swine outside on pasture in the United States (US), instead of inside confinement barns with high levels of biosecurity, has been increasing in the last few decades and is a possible risk factor for the transmission of foodborne disease in the wildlife-livestock-human interface.(62)(38)(195)(196)(122)(123) Generic *Escherichia coli* are commensal bacteria living in all mammals; however, pathogenic Shiga toxin-producing *Escherichia coli (*STEC) strains can cause severe illness in humans and can be transmitted through consumption of contaminated produce and meat from livestock or wild animals. Both feral and domestic swine are considered a species of concern for the transmission of foodborne pathogens, because they are the same taxonomy (i.e., *Sus scrofa*) and therefore can share diseases, and both harbor zoonotic pathogens such as STEC, *Salmonella* spp., *Brucella suis* and swine influenza virus.(62)(145)(18)(197)(16)(17)(14)(127)(47)(63)(198) Information regarding the prevalence of STEC in domestic swine raised outdoors is sparse, and more data is needed to understand whether feral pigs that live near these outdoor swine operations may pose a risk for the transmission of pathogens between these two swine populations.

Referenced in this study as outdoor-raised pig operations (OPO), these type of farms are primarily considered niche market production operations.(38)(199) However, OPO are broadly distributed throughout California, providing an opportunity for the transmission of emerging or reemerging diseases that had been eradicated in confinement swine herds, as each outdoor-based domestic pig could act as an intermediary host between feral pigs and humans. Feral pigs in the US are a mix of introduced Eurasian wild boars and escaped domestically-raised pigs turned feral.(49)(51) Additionally, feral pigs harbor and transmit emerging or transboundary diseases (TBD), such as African Swine Fever and pseudorabies. (61)(134)(200-202) Feral pigs are also

attracted to agricultural areas for food, water and mates, which facilitates direct or indirect pathogen transmission.(203)(204)(63)(187) States at greatest risk for increased contact between feral and domestic pigs and consequential potential disease transmission, are those regions containing large populations of feral pigs living near outdoor-raised domestic pigs reared with low levels of biosecurity.(63)(130)(143)(125) Despite multiple control and eradication efforts, California has one of the largest and widest distributions of feral pig populations, which continues to expand.(34)(139) High-risk contact areas between feral pigs and outdoor-based swine could have an important role in the spread of future emerging or reemerging diseases, including TBD, which could negatively impact California, the top agricultural production state in the US.(10)(47)(126) The risk map built in Chapter 2 determined that more than 40% of the 305 identified OPO in California are situated within suitable feral pig habitat areas.(205)

Both feral and domestic swine are reservoirs for STEC. (18)(206-208) A study identified feral pigs in California as reservoirs of *E. coli* O157:H7, with prevalence ranging from 5.0% to 23.4%, depending upon sample type.(31) A 2018 Georgia study, another state in the US with large feral pig populations, reported 19.5% STEC prevalence in feral pigs.(198) In Chapter 1, Patterson *et al* (2021) reported a STEC prevalence of 5.59% (8/143) in pigs reared outdoors on small-scale diversified farms in California. Moreover, serogroups O26 and O103 were found in positive STEC samples in Chapter 1, which are listed in the top seven serogroups (i.e., O157:H7, O111, O103, O26, O121, O45, and O145) that account for over 95% of human STEC illness in the US.(209) Foodborne STEC infections have previously occurred in humans ingesting contaminated pig products. For instance, outbreaks of *E. coli* O157:H7 occurred in Canada in 2011 and 2014 from consumption of contaminated pork products.(210-212) Although pork is not currently considered a major source of foodborne infection in the US, many studies recognize the

importance of STEC maintained in both domestic pigs and feral swine in the US and internationally.(207)(197)(213-218)

The objectives of this study focused on a) measuring the prevalence of STEC in outdoor-raised domestic pigs and feral pigs located near these OPO, and b) analyzing risk factors for the presence of STEC on OPO that operate near feral pig populations in six high-risk California counties, as determined by the risk map built in Chapter 2.

Materials & Methods

Study design and participant enrollment

We conducted a cross-sectional study between February and August 2018 to collect fecal samples from outdoor-raised domestic pigs and feral pigs in six high-risk California counties. Sample collection was targeted to six high-risk counties that had a higher likelihood of feral pig to outdoor-raised pig contact, based on the risk map built in Chapter 2 and communication with landowners that had feral pig presence on their land. OPO enrollment criteria for this study included 1) reared domestic pigs outdoors in one of the six targeted counties; 2) willingness to participate; and 3) farm owners had seen evidence of feral pigs on their property, or their farm was located near suitable feral pig habitat, according to the risk map built in Chapter 2. Recruitment techniques included personal farm visits, previous working or research connections, farmers markets, and agricultural festivals. Once OPO were enrolled, we identified feral pig locations on or near those farms or at least within the same county. Feral pig locations were identified through conversations with landowners, hunters, University of California Cooperative Extension advisors or United States Department of Agriculture Wildlife Services (USDA-WS) staff, who conduct disease surveillance in feral pigs in some California counties.

Sample collection and questionnaire

We collected domestic swine fecal samples from participating OPO, including ones that also had feral pig presence on their farm. To collect feral pig fecal samples, we worked with landowners to identify known locations on their properties, then we looked for signs of feral pig presence, such as wallowing areas, swine footprints and/or rooting for food. Once feral pig areas were identified, we searched for fecal samples. A feral pig fecal sample was considered authentic if the fecal pile resembled typical pig feces, was surrounded by at least of one of the feral pig presence signs listed above and there were no other livestock within the collection pasture that could contaminate a sample. All study sites were visited once, except for one private ranch that had high feral pig presence throughout the study period and was visited three times.

Based on previous studies, we assumed a STEC prevalence of 5% for outdoor-raised pigs and 10% for feral pigs with a 10% precision error for both, which resulted in needing approximately 204 domestic pig samples and 72 feral pig samples.(219) Sample size per farm was calculated by proportional stratified methods. The total number of samples collected per farm was based on total pig count and the number inside each paddock, pen or pasture. Fresh fecal samples were collected from the ground or from a feral pig's colon, if it had been freshly hunted. Fecal samples were gathered with gloves and placed into sterile cups (MedicusHealth, Kentwood, MI US) with sterilized wooden tongue depressors (Puritan, Guilford, ME US) Samples were placed into a cooler containing ice packs and brought to the lab for processing within 24-48 hrs.

All participants were asked to complete a questionnaire that included topics regarding known nearest feral pig locations, farm demographics and domestic pig health, as related to each type of study participant. The survey instrument and protocols were reviewed by the Institutional Review Board of the University of California-Davis (No. 1180798-1). A directed acyclic graph

(DAG) was built to assess key questions to include in the questionnaire. (91)(92)(220) Once the study participant completed the questionnaire, they were sent a $30 gift card as a thank you gift for assisting us with the study and as a motivation to complete the survey. Participants completed the questionnaire via phone, email or mail. If missing data was identified, then a follow-up call or email was initiated to gather these answers.

Laboratory methods

Fecal sample preparation

All fecal samples were screened for *E. coli* O157:H7 and non-O157 STEC and tested for *stx*1 and *stx*2 genes. Upon arrival to the laboratory, fecal samples were cultured for STEC, using a modified version of a previous protocol.(74) Briefly, for each fecal sample, a Tryptic Soy Broth (TSB) enrichment was performed for detection of non-O157 STEC and *E. coli* O157:H7.(74) Ten grams of fecal material was weighed and added to a pre-refrigerated 24 oz Whirl-Pak sterile bag (Nasco, Modesto, CA) filled with 90 mL of TSB (1:10 dilution) and manually homogenized for one minute. Then samples were incubated in a shaking incubator at 100 RPM and held at 4°C. A Multitron programmable shaking incubator (Eppendorf, Hauppauge, NY, USA) was employed in this study.

Non-O157 STEC enrichment, isolation and confirmation

For non-O157 STEC confirmation, 1 mL of TSB enrichment media was added to 9 mL of modified enterohemorrhagic *E. coli* (mEHEC) selected media (Biocontrol, Bellevue, WA, USA), and then was incubated at 42°C for 12 h with agitation. Next, ten µL were streaked onto CHROMagar STEC (CHROMagar™, Paris, France) with an inoculation loop and the plates were incubated at 37°C for 24 hrs. Up to six presumptive non-O157 STEC colonies were selected from the primary plates and sub-streaked to secondary and tertiary plates.(74) Presumptive

positives were plated onto Tryptic Soy Agar (TSA) and subjected to PCR for confirmation, which tested for *stx*1 and *stx*2 genes.(221) Up to six isolates were banked per sample in a 15% glycerol and 85% TSB solution and stored in a -80°C freezer. (For more details see Ramos *et al* 2019*)*.(222)

E. coli O157:H7 STEC enrichment, isolation and confirmation

One mL of TSB enrichment media for each sample was subjected to immunomagnetic separation (IMS) to concentrate *E. coli* O157:H7 on CT-SMAC (BBL™; Sorbitol MacConkey II Agar 285with Cefixime and Tellurite) and RainbowAgar O157:H7 with novobiocin (Biolog, Hayward, CA) using previously described protocols.(74)(97)(223) Up to four presumptive *E. coli* O157:H7 colonies per sample (two per media) were selected for PCR confirmation using the PCR protocol outlined previously for *E. coli* O157:H7 detection.(224) Positive samples were banked in a 15% glycerol and 85% TSB solution and stored in a -80°C freezer.

Whole genome sequencing to identify virulence genes and serotypes

DNA Extraction and Purification for WGS

STEC isolates were sent for whole genome sequencing (WGS) to the University of California, Davis Genome Center, after PCR confirmation, clean-up and DNA extraction. Briefly, isolates were grown overnight aerobically at 37°C in autoclaved 15 mL culture tubes containing 10 mL Brain Heart Infusion broth. DNA was then extracted according to the DNeasy Blood and Tissue Kit (Qiagen N.V., Carlsbad, CA). To ensure adequate purity for DNA sequencing, eluted DNA was purified according to the Zymo Quick-DNA Miniprep Kit (Zymo Research, USA). 30 µL of purified DNA was eluted for each isolate into a sterile 2 mL micro centrifuge tube. DNA quantification was conducted for all isolates using a NanoDrop One^{C} (Thermo Fisher Scientific, Wilmington, DE). DNA samples were stored at -80°C.

Whole genome sequencing and bioinformatics

The raw reads were pre-processed using HTStream (version 1.3.2)(225) to remove contamination, remove duplicates, overlap reads, and trim based on quality and length. Next, the processed reads were assembled using SPAdes (version 3.14.1).(226) BUSCO (version 4.1.4)(227) was utilized to check assembly completeness and find common single-copy orthologs across all of the isolates. Using common sequences, "pseudo-genomes" were created for each isolate, after which mafft (version 7.471)(228) was used to create a multiple alignment across all of them. The final analysis used VirulenceFinder (version 2.0)(229) to identify virulence genes for all of the isolates. Custom R (version 4.0.1)(230) code was used to collate the results of each of the finders into a superset of the hits for each of the analyses. Using the supersets, heatmaps were generated across all the isolates and genes.

Risk factor analysis and model building

Descriptive analysis of variables was conducted, including calculating STEC prevalence (number of positive samples divided by total number of samples collected per sample category) at the farm-level and county-level with a 95% confidence interval (CI_{95}). Sampling day weather data was resourced from online sites, including the California Irrigation Management Irrigation systems (CIMIS) weather stations within a similar microclimate (http://www.cimis.water.ca.gov) and National Oceanic and Atmospheric Administration, National Centers for Environmental Information (NOAA NCEI) (https://www.ncdc.noaa.gov/cdo-web/datatools/findstation).(88)(231) Distance between each OPO and the nearest surface water or wild area was measured using GoogleEarthPro to estimate suitable resources for feral pigs nearby.(185)

Data analysis was conducted using generalized linear mixed models (i.e., logistic

regression with farm as a random effect) to identify significant risk factors for the presence of STEC in outdoor-raised pigs (i.e., binary outcome STEC presence yes or no). Univariate analysis was used to initially assess the distribution of variables. During bivariate analysis, variables with low variability, small cell sizes (<5), or large standard errors were either modified, collapsed if appropriate, or discarded from model building. Correlations between variables were ascertained using the Spearman's rank correlation coefficient. Possible confounders were identified using a directed acyclic graph (DAG) and then included in models to assess significant changes in the odds ratio. The sample size in this study was too small to detect effect modifiers.

Manual two-way stepwise variable selection was employed for model building, using add1 and drop1 functions in the stats R package. Models were built using the glmer function from the lme4 package, with farm as a random affect.(95)(93) Variance inflation factors measured multicollinearity within each model. Top models were compared, and a final model was chosen based on the lowest Akaike Information Criterion (AIC) and smallest deviance. Model diagnostics were conducted on final models using the DHARMa package.(94) Intraclass correlation (ICC) was calculated. Odds ratios and 95% confidence intervals (CI_{95}) were calculated for all variables in the final model. All data analysis was performed using R Statistic Software version 1.4.1036 ©.(95)

Results

Study participants

The entire study included 17 farms or ranches, but not including the two feral pig samples collected by USDA-WS, of the remaining 16 participants: 56.25% (9/16) were diversified farms (i.e., those that raised a mix of livestock and crops); 18.75% raised multiple types of livestock, but no crops (3/16); 12.5% reared pigs only (2/16); and the remaining two ranches, (12.50%)

were private landowners who did not raise domestic pigs, but had feral pig presence on their land (2/16). Of the 16 participants, 62.50% (10/16) also raised poultry, 25.00% (4/16) sheep, 31.25% (5/16) cattle, 31.25% (5/16) goats, and 12.5% (2/16) equine. Domestic swine diseases reported by farmers during the 2018 study period included pneumonia, diarrhea, PRRS and a non-diagnosed respiratory condition; each of these was reported on one farm each.

Fourteen of the 16 questionnaires were completed, of which 11 were for OPO. The two incomplete questionnaires belonged to the two participants who only raised swine, not crops or other livestock. None of the answering 11 OPO had direct bordering neighbors who raised domestic swine. Five of the 11 OPO (36.36%) reared their domestic swine outdoors with access to wild areas (e.g., forest, rangelands) and approximately half of the responding 11 OPO (54.54%; 6/11) allowed visitors direct contact with pigs.

Five of the 11 OPO (45.45%) had seen evidence of feral pig presence on their farm (e.g., animals, rooting, wallowing) with four (36.36%) stating that feral pigs had direct contact (i.e., within 100ft) with their domestic pigs in pastures, pens or barns. Of the five that had feral pig presence on their farms, three witnessed feral swine monthly and two observed them on a weekly basis. The number of feral pigs reported per farm owner ranged from a minimum of one up to 100 pigs and the maximum number observed ranged from 15 up to 300. Of the remaining six OPO with no feral pig presence on their farms, five (45.45%) had seen feral pigs in their counties less than five miles from their operation and only one OPO (9.09%) had never seen signs of feral pigs in their county.

Of the six targeted counties in this study, we enrolled one to four OPO and one to three feral pig locations per county. Although we were able to enroll OPO in each of the targeted six

counties, feral pig samples were unattainable in San Mateo, Nevada and Monterey counties. Feral pig samples were collected in Mendocino, Yolo, and Sonoma.

STEC prevalence and serotypes

A total of 192 fecal samples were collected during the 2018 study period: 43 feral pig samples and 149 outdoor-raised pig samples. Mean number of fecal samples collected at each farm was eleven with a median of ten. Feral pig fecal samples were collected on eight properties with a sample number range of 1-14 per location.

Regarding STEC prevalence on the 13 participating OPO, at least one STEC positive sample was collected on 61.54% (8/13) farms. STEC prevalence per farm ranged from 0% to 83.33% with a mean of 23.00% and median of 20.00%. At the county level, overall STEC prevalence for feral pigs ranged from 0% to 25.00% and 0% to 57.14% for domestic outdoor-raised pigs (Table 3.1). Overall, 35 out of the total 192 samples (18.23%) were positive for STEC, with five samples collected from feral pigs (11.63%; 5/43) (CI_{95} = 5.07% - 24.48%) and 30 samples gathered from domestic pigs (20.13%; 30/149) (CI_{95} = 14.48% - 27.29%). Results for *E. coli* O157:H7 included one positive feral pig sample (2.33%; 1/43) (CI_{95} = 0.12% - 12.06%) in Yolo County and three *E. coli* O157:H7 positive domestic pig samples (2.01%; 3/149) (CI_{95} = 0.69% - 5.75%), one from Yolo County and two from Sonoma County. Non-O157 STEC positives included four feral pig samples (9.30%; 4/43) (CI_{95} = 3.68% - 21.60%) and 27 domestic pig samples (18.12%; 27/149) (CI_{95} = 12.76% - 25.08%).

Table 3.1: Shiga toxin-producing *Escherichia coli* (STEC) O157:H7 and non-O157 STEC prevalence and the number of positive samples per swine category (feral or domestic), and county, from a cross-sectional study conducted in 2018 in California, from 17 study participants.

County	# Total samples	# non-O157 positive	# O157:H7 positive	Prevalence non-O157	Prevalence O157:H7	Total All STEC Prevalence
FERAL PIGS						
Mendocino	21	0	0	0.00%	0.00%	0.00%
Yolo	16	3	1	18.75%	1.17%	25.00%
Sonoma	5	1	0	20.00%	4.00%	20.00%
Monterey	1	0	0	0.00%	0.00%	0.00%
Nevada	0	NA	NA	NA	NA	NA
San Mateo	0	NA	NA	NA	NA	NA
All Feral Pigs	**43**	**4**	**1**	**9.30%**	**2.33%**	**11.63%**
OUTDOOR-RAISED DOMESTIC PIGS						
Mendocino	25	0	0	0.00%	0.00%	0.00%
Yolo	36	10	1	27.78%	2.78%	30.56%
Sonoma	39	2	2	5.13%	5.13%	10.26%
Monterey	26	3	0	11.54%	0.00%	11.54%
Nevada	7	4	0	57.14%	0.00%	57.14%
San Mateo	16	8	0	50.00%	0.00%	50.00%
All Domestic Pigs	**149**	**27**	**3**	**18.12%**	**2.01%**	**20.13%**
				All Pigs non-O157	**All Pigs O157**	**Overall STEC Prevalence**
GRAND TOTALS (Feral + Domestic)	***192***	***31***	***4***	***16.15%***	***2.08%***	***18.23%***

Table 3.2: Serotypes and virulence factors detected by whole genome sequencing in 25 of 35* Shiga toxin-producing *Escherichia coli* isolates collected from feral pigs and domestic swine raised outdoors from participating ranches in California during 2018.

Swine Source	Feral pigs nearby?	Serotype	*ehxA*	*eae*	*stx2*	*stx1*
Domestic	on farm	O8:H21	-	-	-	-
Domestic	on farm	O5:H11	-	-	-	-
Domestic	on farm	O26:H11	-	+	-	+
Domestic	on farm	O26:H11	-	+	-	+
Domestic	on farm	O26:H11	+	+	-	+
Domestic	w/in 3.22 km	O26:H11	+	+	-	+
Domestic	w/in 3.22 km	O26:H11	+	+	-	+
Domestic	none	O26:H11	+	+	-	+
Domestic	none	O26:H11	+	+	-	+
Domestic	on farm	O178:H19	-	-	-	+
Domestic	on farm	O154:H32	-	-	-	-
Domestic	on farm	O153:H4	-	-	-	-
Domestic	w/in 3.22 km	O141:H29τ	-	-	+	-
Domestic	w/in 3.22 km	O141:H29τ	-	-	+	-
Feral pig	NA	O118:H16	+	+	-	+
Feral pig	NA	O118:H16	+	+	-	+
Domestic	none	O100:H20	-	-	+	-
Domestic	none	O100:H20	-	-	+	-
Domestic	none	O100:H20	-	-	+	-
Domestic	none	O100:H20	-	-	+	-
Domestic	on farm	O103:H11	+	+	-	+
Domestic	on farm	:H34	-	-	-	-
Domestic	w/in 3.22 km	:H26	-	-	-	-
Domestic	on farm	:H21	-	-	+	-
Domestic	w/in 3.22 km	:H21	-	-	+	-

**Ten positive isolates were not sent for whole genomic sequencing, but four of those were identified as O157:H7 in our lab: three from domestic pigs and one from a feral pig but these four O157:H7 are not included in the table.*
+ indicates presence, - indicates absence
τ Also contained O141ab and O141ac

Table 3.2 shows the serotypes and virulence factors identified in 25 of the 35 total positive STEC samples sent for WGS. Ten isolates were not sent for WGS, but four of those were reported as *E. coli* O157:H7 in our laboratory. *Stx2* was detected in 32.00% (8/25) and *stx1* in 44.00% (11/25) of sequenced isolates. Of the 25 sequenced isolates, seven (28.00%) were O26:H11, which all contained *stx1* but not *stx2*. The *ehxA* gene was detected in 32.00% (8/25) of sequenced isolates and *eae* found in 36.00% (9/25). Six samples did not indicate presence of Shiga-toxin genes (i.e., *stx1* and *stx2*). Two samples were identified as both O118 and O151.(232)

Risk Factor Analysis

Variable selection for generalized linear mixed model building began with over 80 factors, including sampling day data (e.g., ambient humidity, season), individual or pen-level characteristics related to the collected fecal sample (e.g., sex, age) and questionnaire variables. Table 3.3 showcases mean, median and range of selected numeric demographic variables. For instance, the size of domestic swine herds for the 11 OPO ranged from 3 - 350 pigs (mean=72; median = 35). Stocking density ranged from 0.67 to 106.67 pigs per acre, based on total area dedicated to pigs on each farm (mean = 17.44 pigs/acre; median 7.0 pigs/acre). The average number of acres per OPO participating in this study was 508.12 with a median of 62.00 acres. Enrollees had been farming for a mean of 14.73 years and median of 12.00 years but had been raising domestic pigs for a mean of 8.18 years, with a median of 6.00 years.

Table 3.3: Mean, median and range (i.e., minimum and maximum) of selected numeric factors assessed for model building and collected during a cross-sectional study conducted in 2018 from 11 outdoor-raised pig operations in California.

Description	Mean	Median	Minimum	Maximum
Density (#pigs/acre)	17.44	7.00	0.67	106.67
Total number of pigs raised per farm	72	35	3	350
Number years farm has been in operation	14.73	12.00	1.00	55.00
Number years farm has been raising pigs	8.18	6.00	1.00	25.00
Total farm acres	508.12	62.00	5.00	2200.00
Total acres used for outdoor-raised pigs	35.55	5.00	0.10	300.00
Mean pig herd size	84	35	3	350
Distance to nearest surface water (km)	1.14	0.51	0.10	5.07
Distance to nearest wild area (km)	1.08	0.66	0.13	5.84
Nearest known feral pig location (km)	8.39	2.32	0.14	24.00

Table 3.4 displays selected categorial variables stratified by positive (n=24) and negative STEC samples (n=112) for the 11 OPO that finished their questionnaire. P-values were reported with chi-square test or Fisher's Exact test if cell sizes were less than five. Regarding type of operation, 87.50% (21/24) of the positive samples were collected on diversified OPO (i.e., raised livestock and crops) vs. 12.50% (3/24) from farms rearing multiple types of livestock, but no crops. All the 24 positive STEC samples were collected in the spring, none of the STEC positive samples were gathered in the summer months. Most positive samples (70.83%; 17/24) were isolated from juveniles (i.e., aged 1-11 months and weaned). Most of the STEC positive samples (79.17%; 19/24) were from farms that raised their domestic pigs with access to wild areas (e.g., forests, riparian areas). Most positive samples (87.50%; 21/24) were from farms that do not use antimicrobials, either in feed or as injectables.

Table 3.4: Characteristics of selected categorical variables stratified by positive or negative Shiga toxin-producing *Escherichia coli* (STEC) status during bivariate analysis. Fecal samples were collected during a cross-sectional study conducted in 2018 on 11 outdoor-raised pig operations in California.

Description	Levels	STEC negative ct (%)	STEC positive ct (%)	p-value
Type of farm	Diversified [a.]	67 (59.82%)	21 (87.50%)	0.019
	Multiple livestock	45 (40.18%)	3 (12.50%)	
Sample season	Spring (Feb-April)	74 (66.07%)	24 (100.00%)	< 0.001
	Summer (May-August)	38 (33.93%)	0 (0.00%)	
Age of pig fecal source	Adult (12 months or older)	57 (50.89%)	3 (12.50%)	0.001
	Juvenile (> 1-11months)	44 (39.29%)	17 (70.83%)	
	Piglets (on sow)	11 (9.82%)	4 (16.67%)	
Does farmer use antimicrobials for pigs?	Yes	36 (32.14%)	3 (12.50%)	0.093
	No	76 (67.86%)	21 (87.50%)	
Source for swine drinking water	Surface	53 (47.32%)	12 (50.00%)	0.989
	Private well	59 (52.68%)	12 (50.00%)	
Is there direct contact between feral pigs & domestic pigs?	Yes	58 (51.79%)	14 (58.33%)	0.720
	No	54 (48.21%)	10 (41.67%)	
Where have you seen evidence of feral pigs?	On-farm	61 (54.46%)	14 (58.33%)	0.038
	None	20 (17.86%)	0 (0.00%)	
	In the same county	31 (27.68%)	10 (41.67%)	
Does farm allow domestic swine access to wild areas?	No	57 (50.89%)	5 (20.83%)	0.012
	Yes	55 (49.11%)	19 (79.17%)	
New or long-time farmer	Long	74 (66.07%)	16 (66.67%)	
	New	38 (33.93%)	8 (33.33%)	

[a.] Diversified farms raise a combination of livestock and numerous types of produce

Final multivariable model results

The final mixed effect multivariable logistic regression model is shown in Table 3.5 and was chosen based on the lowest AIC and smallest deviance, relative to other models. The AIC was 97.9 and deviance was 83.9. The highest variance inflation factor for any variable in the final model was three, which was below our threshold of five. The ICC was 0.09, which accounts for the proportion of the model variance explained by clustering by farm and indicated that including the farm as a random effect was necessary. The isSingular test function in the lme4 package was false, which indicated that no singularities existed in the final model.

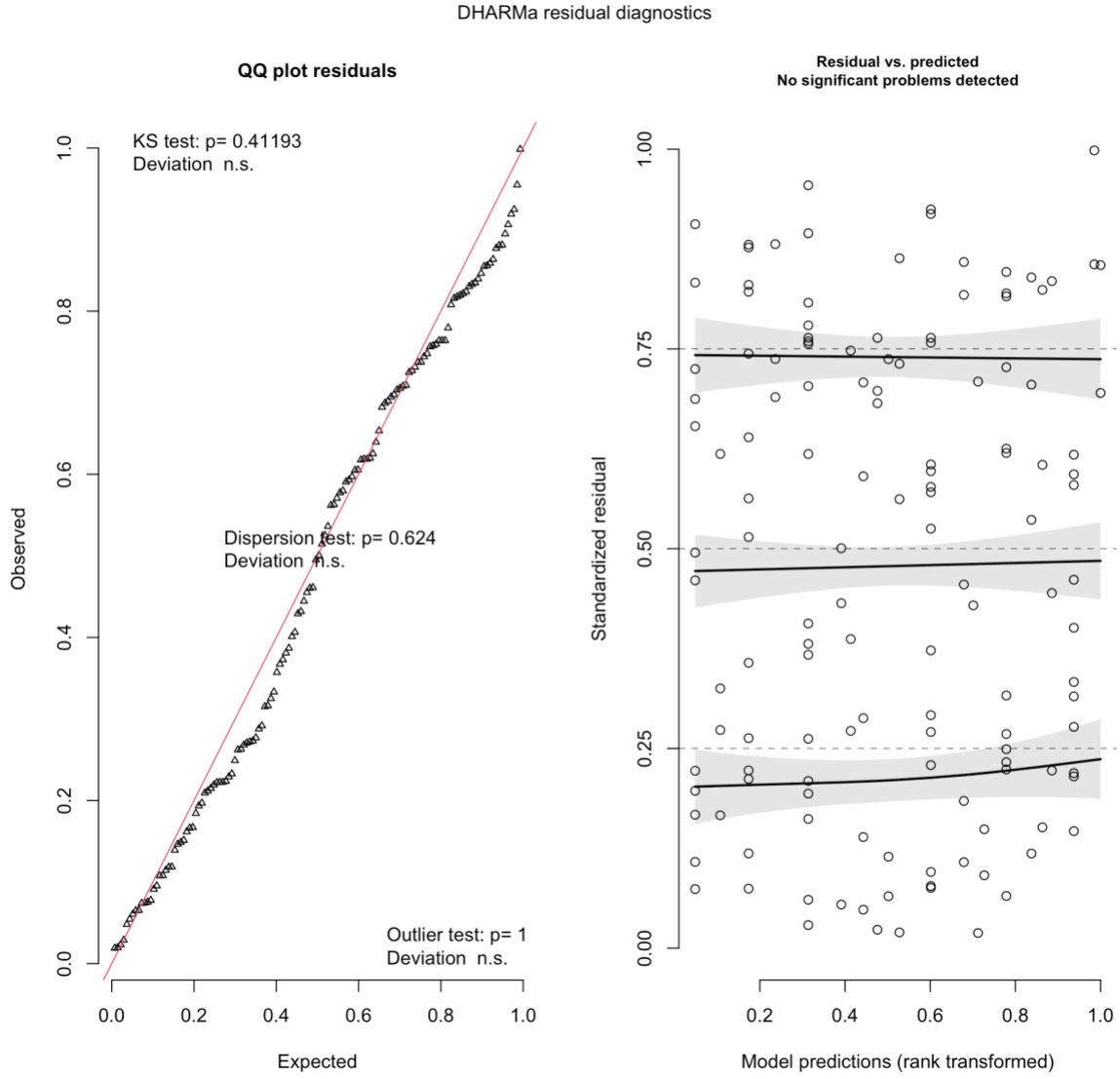
Table 3.5: Association between the presence of Shiga toxin-producing *Escherichia coli* in swine fecal samples and risk factors as determined by a multilevel logistic regression model, from data gathered in California between February and August 2018.

Variable	Level	Estimate	OR	OR 95% CI	p-value
Intercept		*-3.29*			*< 0.001**
Pig age group	Adult (12 months or older)	*reference*			
	Juvenile (> 1-11months)	2.16	8.66	2.11 - 47.89	0.005*
	Piglets (on sow, not weaned)	1.55	4.73	0.71 - 33.88	0.102
Nearest surface water	*numeric*	*-3.43*	0.03	0 - 0.23	0.020*
Pig allowed access wild areas?	*No*	*reference*			
	Yes	2.90	18.24	2.94 - 520.31	0.013*
Years farming	*6-30 years*	*reference*			
	1-5 years	1.84	6.31	0.76 - 148.06	0.100

** Denotes statistical significant at the 0.05.*

Diagnostically, the simulated residuals versus predicted values did not show any significant problems: the Q-Q plot of DHARMA simulated scaled residuals was linear with no major deviations, and the Kolmogorov-Smirnov test indicated no deviation from uniform distribution of the scaled residuals (see Figure 3.1). The adjusted ICC was 0.09 for the final model, which signifies the proportion of the variance that is explained by farm clustering and indicated the need for a farm random effect.

Figure 3.1: Q-Q plot of residuals (left) and residuals versus predicted values plot (right) from the DHARMa package in R.



Significant variables identified in the final multivariable logistic regression model were the pig age group of the sample source, distance to the nearest surface water, and if the farm raised pigs in an area that had access to a wild area (yes/no). Whether the farm was new (farmed < 5 years) or had been farming for a longer period (> 6 years) was adjusted for as a confounder in the final model according to a DAG but was not significant (p-value = 0.10). On average, while holding other effects constant, for every one km increase in distance between the OPO and nearest surface water, the odds of a STEC positive sample decreased (OR = 0.03; CI_{95}: 0.00-0.23). The odds of a STEC positive sample are 18.24 times higher on farms that allowed domestic pigs access to wild areas than farms that did not (OR = 18.24; CI_{95}: 2.94-520.31). The odds of a STEC positive sample were more than eight times greater for a fecal sample collected from a juvenile pig (OR = 8.66 ; CI_{95}: 2.11-47.89), than adults.

Discussion

This study determined STEC prevalence for feral pigs and domestic pigs raised outside near feral pig locations in high-risk California counties and assessed risk factors associated with the presence of STEC on OPO. We estimated an overall STEC prevalence in outdoor-raised pigs of 20.13%, and 11.63% in feral pigs. Also, serotypes implicated in severe human disease were identified in fecal samples through WGS. Significant risk factors associated with STEC presence on OPO included age of swine sampled (i.e., juveniles), the distance to the nearest surface water, and whether a farm raised domestic swine with access to a wild area (e.g., forest, wetlands).

The non-O157 STEC prevalence in this study was 18.12% in domestic pigs reared outdoors and 9.30% in feral pigs. *E. coli* O157:H7 results were 2.33% in feral pigs and 2.01% in domestic pigs. Prevalence of *E. coli* O157:H7 and non-O157 STEC in swine varies greatly worldwide, and US studies measuring STEC in OPO are sparse. Although STEC was identified

in outdoor raised domestic swine in both Chapters 1 and 3, the overall prevalence was much larger in Chapter 3 versus Chapter 1 (i.e., 5.59%). Prevalence of STEC in domestic pigs reared outdoors on diversified small-scale farms from Chapter 1 was lower than this current study, but had a similar sample size (i.e., n=143 vs n=149 in Chapter 3). Samples were collected in 2018 for Chapter 3 and 2015-16 for Chapter 1. Differences in STEC prevalence between 2015-2016 and 2018 may be due to different laboratory processing methods or environmental factors. Both study periods were drought years in California; however, 2017 was a very wet year, which may have affected 2018. Three farms participated in both studies and all three farms saw increases in STEC prevalence between 2015-16 and 2018: Farm 1 had a 5.13% (2/29) STEC prevalence in 2015-16 compared to 20.00% (6/30) in 2018, Farm 2: 0% (0/15) STEC prevalence increased to 83.33% (5/6) and Farm 3: 11.11% (1/9) to 66.67% (2/3). However, a smaller number of samples and animals for Farm 2 and 3 accounts for some of this seemingly large increase between studies. A 2018 study conducted in Georgia reported 62.5% (5/8) STEC in organic "free-ranging" domestic swine, but reported a small sample size of eight.(198) Differences between STEC prevalence may be due to different study designs, laboratory tests, environmental factors or farm management practices, such as the density of pigs raised in each paddock. STEC has been identified in indoor raised swine herds but comparison studies are lacking.(233)(19) The scarcity of data regarding STEC in swine raised outdoors indicates a need for future studies.

Studies measuring the prevalence of STEC in feral pig populations in the US are infrequent, unlike European studies.(234)(58)(235) A 2006 US study sampled swine necropsy and fecal samples and reported 0 - 23.4% prevalence *E. coli* O157:H7 in feral pigs.(31) A 2018 study conducted in Georgia detected an overall STEC prevalence of 19.5% (17/87) in feral swine and they identified a higher prevalence of STEC in feral pigs sampled in agricultural

counties.(198) Feral pigs are attracted to agricultural areas because of resource availability (e.g., fresh produce, livestock feed, mates), and their direct or indirect contact with livestock may create a risk of foodborne pathogen transmission. The risk of pathogen sharing between feral pigs and domestic swine has been studied, but only a small subset of these studies investigated the risks to outdoor based pigs, even though there have been multiple cases of feral pigs transmitting pathogens, such as *Brucella suis,* to domestic swine raised outdoors.(46)(56)(59)(60) Wyckoff *et al* (2009) concluded that increasing populations of feral swine are a risk for the reintroduction of eradicated diseases as well as emerging TBD, especially for backyard operations that allow domestic swine outdoor access, because male feral pigs are attracted to female pens.(56) In a Corsica study that focused on traditional pig farms that raise their animals outdoors, the authors determined that a significant risk factor for the spread of diseases between wild boars and domestic swine was interactions between these two swine groups.(236) Our study results indicated that 45.45% of farm participants had seen evidence of feral pig presence on their farms. Schembri *et al* (2015) conducted a questionnaire of backyard and small-scale swine producers in Australia and found that a third of producers, both indoor and outdoor, had seen feral pigs on their farms.(125) Understanding the prevalence of STEC in feral pigs, combined with the aforementioned study results indicating that these animals reside near resource-rich farms, highlights the need for further studies to address the risk of disease transmission associated with feral pig presence near operations that raise swine outdoors.

Serotypes identified in this study that can cause severe human illness included *E. coli* O157:H7 (n=4), O26:H11 (n=7) and O103:H11 (n=1). (209) The serogroups O26:H11 and O103:H11 contained only the *stx1* gene, not *stx2*. The only O103:H11 serotype contained both *eae* and *ehxA* and all the O26:H11 isolates contained the *eae* gene, with five (71.43%) O26:H11

serotypes also containing the *ehxA* gene. A study by Cha *et al* (2018) also found O26 with *stx1* and *eae* in commercial swine raised indoors in Ohio, US. (207) A study conducted in finishing swine, measured 6.9% of positive samples were O26 and 2.4% contained O103. (237) In 2017, the US Food Safety and Inspection Service (FSIS) conducted a Raw Pork Baseline Study to determine the prevalence of STEC in various types of pork products at slaughterhouses and processing facilities and measured a prevalence of 0.2% STEC, mostly in comminuted pork products.(214) However, this study only looked for the top seven STEC serogroups, even though 309 other samples were positive for key virulence factors like *stx* and *eae* genes.(214) Additionally, on-farm or slaughterhouse swine samples may reflect different prevalence ranges than meat products. Considering most studies identified *E. coli* O157:H7 and non-O157 STEC serotypes that cause human illness in swine samples, pigs should be considered an important reservoir of STEC, and mitigation strategies established to prevent the spread of foodborne pathogens from farm to consumer.

Significant risk factors associated with the presence of STEC in fecal samples collected during this study included distance from the nearest surface water and whether domestic swine had access to wild areas, such as forest or wetlands. These variables were measured as a proxy for suitable feral pig habitat that borders farms.(61)(47)(58)(238-240) Feral pigs are reservoirs of STEC, and surface water and/or wild areas provide habitat for these animals to exist near OPO.(62)(63)(203)(204) For instance, a study by Rutten *et al* (2019) predicted suitable habitat for wild boar in Belgium and identified forest (e.g., coniferous and deciduous), as a significant predictor.(146) Additionally, Wu *et al* (2012) reported distance from a forest to be a significant risk factor for contact with wild boars in Switzerland, especially those domestic pigs less than 500 meters from a forest.(62) A 2017 study reported that distance to water affects feral pig

movement mostly in states where water is scarce (e.g., Texas, California) versus states where water is more prevalent (e.g., Georgia, Louisiana).(187) Additionally, feral pigs may contaminate these habitat areas, which may lead to indirect STEC transmission to swine raised outdoors, as studies have shown that STEC can be transmitted through contaminated surface water sources and the environment. (187)(241)(242) A 2014 study conducted in the Central Coast of California detected *E. coli* O157:H7 and non-O157 in many water sources.(242) These results indicate a need to separate domestic swine raised outdoors from wild areas to avoid direct or indirect transmission of pathogens from feral pigs.

In this current study, only the juvenile age group, which included weaners, finishing and market swine (i.e., ages 1-11 months), was significant when compared to adults. Many US and international studies have tested similar-aged pigs at slaughterhouses and reported a wide range of STEC prevalence.(208)(243) A study by Tseng *et al* (2015) sampled finishing pigs (i.e., aged 10-24 weeks), which are included in our juvenile category, and determined that the highest prevalence (i.e., 39.5-59.2%) amongst three cohorts occurred between 14-18 weeks of age.(197) At 24 weeks, STEC prevalence in all cohorts had dropped and ranged from 0 - 6.7% in the three groups. (197) This same study mentions that the finishing age group are most susceptible to STEC oedema, which is caused by *E. coli* strains carrying the *stx2* gene and may be associated with detecting STEC in this juvenile age category. A longitudinal study conducted by Cha *et al* (2018) in commercial indoor domestic swine found that 68.3% (82/120) of finishing pigs (i.e., aged 10-24 weeks) shed STEC at least once during the study period, which showcases the intermittent nature of STEC shedding in swine.(207) The high prevalence (i.e., 68.3%) identified in this study might be due to repeated sampling over a longer period of time than conducted in our study. Additionally, our study sampled all ages of swine only once, which might indicate an

underreporting of STEC in our results. The effect of age on STEC shedding is more frequently reported in cattle versus swine.(244)(245) For instance, a Raies *et al* (2016) study sampled beef cattle and reported that STEC prevalence was highest during the first six months of life and then decreased toward adulthood.(244) Another study by Cho *et al* (2013) also detected that calves over one month old were two times more likely to shed STEC than those younger than one month, except for pre-weaned calves.(245) If age is a risk factor for STEC shedding in swine, then targeting key age groups for STEC mitigation strategies to reduce the overall bacterial load in slaughtered swine may reduce the risk of these pathogens in the food supply.

Limitations of this study included a small sample size for the total number of farm participants as well as the final number of feral pig samples collected, as we could only gather feral pig feces in three of the six targeted counties. The post-hoc power calculation results were 0.12 for feral pigs and 0.69 for OPO, which indicated that the prevalence estimates are inexact. Moreover, many of the significant variables in the final logistic regression model had wide confidence intervals, which indicates less precise estimates. Since this was a cross-sectional study conducted only during two seasons and only one season per farm, we may have missed STEC positive farms due to seasonality of shedding or other factors that affect STEC detection in feces, including the intermittent nature of shedding in pigs. Our study participants volunteered and therefore we could not conduct random sampling; our study results contain selection bias and are not generalizable to other OPO in California or the US.

Strengths of our study included measuring STEC in both feral pig and outdoor reared pigs in California. This study is an innovative approach toward evaluating areas of contact between feral and domestic pigs reared outdoors, by targeting STEC surveillance based on a risk map built in Chapter 2. Moreover, assessing STEC prevalence in feral pigs near OPO serves as a

proxy for the risk of exposure and transmission of other zoonotic pathogens to domestic pigs reared outdoors. Future research studies could enhance our current study results by comparing STEC strains between the two swine groups using WGS bioinformatic analyses. Similarity of STEC isolates can be used as a biological indicator to track possible transmission of diseases between feral and outdoor-raised swine, as noted in a few recent studies.(62)(145)(204)(37)(45)

Conclusion

Feral and domestic swine are reservoirs of foodborne pathogens like STEC and other zoonoses. Raising domestic pigs outside provides an opportunity for direct and indirect contact with feral pigs that may harbor these pathogens. This study is significant because it will provide a foundation to design targeted, cost-effective disease surveillance and foodborne pathogen mitigation strategies for those areas at highest risk for feral to domestic pig contact. Furthermore, study results will provide a framework to create outreach extension programs to inform stakeholders that raise domestic swine outdoors about disease risks associated with feral pigs. As the number of OPO continues to grow, evaluating risk factors that are unique to these operations will help identify biosecurity practices and develop extension outreach materials to keep food safe from farm to fork and protect California's agricultural economy.

Acknowledgements: The authors would like to thank all the farmers, ranchers and government agency staff that assisted with this study. Also thank you to Pires Lab staff who assisted with sample collection and processing: Viktoria Haghani, La'Chia Harrison, and James Stover.

Funding: This project was supported by the Agriculture and Food Research Initiative grant no. 2019-67011-29609 / project accession no. 1019249 from the USDA National Institute of Food and Agriculture and a CFAH grant.

Dissertation conclusion

The three scientific research projects in this dissertation added important epidemiological information to the body of knowledge regarding STEC detected on DSSF in California and the risk of potential disease transmission from suitable feral pig habitat located near domestic pigs raised outdoors. Although consumers perceive small-scale farms or outdoor-raised meat as safer and more natural, these studies together demonstrate that even livestock raised outdoors on small-scale farms are reservoirs for STEC, including serogroups that cause severe illness in humans, including O157:H7, O26, O103 and O111. Interestingly, Chapter 1 and 3 models indicated that livestock raised outdoors that have access to wild areas, such as wetlands or forest, was a key risk factor for the presence of STEC. Chapter 2 results revealed that nearly 50% of domestic pigs raised outdoors are located near suitable feral pig habitat, and this overlap of feral and domestic swine could be a risk factor for potential emerging or reemerging disease transmission. Also, STEC was detected in domestic swine in both Chapter 1 and 3, even though pigs are currently considered a low risk key species for STEC outbreaks by the US FSIS. These study results indicate the need for further studies on DSSF to ascertain risk factors for foodborne pathogens.

The objective of Chapter 1 entailed conducting an overall assessment of prevalence and risk factors of STEC on diversified small-scale farms in California, while also describing the unique characteristics of DSSF. Temperature was a key risk factor identified in the final multilevel logistic regression model. Many foodborne pathogen studies indicate season as a risk factor for STEC, however, seasons vary across the US. For instance, California summers are characterized by dry heat whereas summers in most states are humid and hot.(89) Measuring and monitoring

temperature during field sampling may be a more precise indicator of risk than season and allow for more accurate comparisons between studies. Additionally, as weather patterns shift due to climate change, assessing environmental factors, (e.g., humidity and temperature), as a risk factor for the presence of STEC on farms will be useful for stakeholders, to understand how weather affects the presence of foodborne pathogens in livestock raised on DSSF. Studies elucidating whether ambient temperature affects survival of STEC in a farm environment or whether temperature affects the host animal harboring STEC will be useful, especially as extreme climate events become more common. Future research projects could also benefit from a larger sample size to analyze farm-level differences and whole genome sequencing (WGS) of positive isolates to ascertain any genetic relatedness between livestock species or between farms, as well as presence of antimicrobial resistance genes.

In Chapter 2, the risk map determined regions at highest risk for contact between feral pigs and outdoor-raised pigs, and these areas will be important to target surveillance and outreach, in the case of future disease transmission between these two swine groups. This project can be expanded nationwide to create awareness of high-risk contact and potential disease transmission areas, to protect both public health and agriculture in the US. For example, this risk map could be used to plan surveillance programs to prevent transmission of imported diseases to the US, such as African Swine Fever (ASF) that was recently detected in the Dominican Republic, or prevent the spread of reemerging diseases like pseudorabies, which was detected in feral pig populations in Mendocino County, CA in 2015. A 2015 report by the European Food Safety Authority (EFSA) concluded that surveillance programs will be key in preventing the introduction and spread of ASF in North America and other naïve countries.(246) Additionally, if feral or outdoor-based domestic swine disease location data were readily accessible in the US, additional

risk maps could predict the spread of specific pathogens. Disease risk maps are useful to support decision making for agencies focused on wildlife management and conservation as well as animal and public health. Currently, USDA collects data through surveys for swine operations only in the top 16 producing pork states in the US, and California is not included, which reduces available data for disease risk models. However, the swine NAHMS is due to be conducted again in 2021 with a larger focus on small-scale swine operators.

Another avenue for future research entails using covariate rasters from specific years or decades to predict the distribution of a feral pigs. Few SDM or MaxEnt comparison projects have compared temporality of covariate rasters for model prediction accuracy. Currently available rasters are limited and are not available for individual years. Shifts in weather patterns, as well as the dynamics of large fires, will most likely be exacerbated by climate change and will affect wildlife movements and locations in the future, which may indicate the importance of choosing temporally-specific variables for model building.(151)(192)(140)(193)(247-249) Additionally, if climate change and wildfires accelerate in California, current static rasters may become inaccurate in predicting future suitable habitat. California's annual precipitation levels fluctuate between drought and excessive precipitation associated with El Niño and La Niña events. Climatologists predict volatility of rainfall patterns and temperature for California, which may affect suitable habitat for wild mammals such as feral pigs, and emphasizes the need for dynamic climate rasters.(192)(249)

Both the MaxEnt model and risk map in Chapter 2 are limited because they are static maps that used fixed layers as their foundation; consequently, they do not incorporate dynamic events. Future species distribution models could incorporate temporal environmental patterns into models, due to dynamic changes over time, especially in regions like California where

climate change and large wildfires can affect the distribution of certain species.(151)(192)(140)(249) Extreme weather events can alter feral pigs' normal sedentary patterns.(48)(151) For instance, Snow et al (2017) used temporally dynamic prediction models and examined three decades from 1982 to 2012 to report evidence of feral pig expansion due to climate change**.** Also, feral pigs in Canada build "pigloos" to be able to survive the harsh Canadian winters, expanding their habitat northward.(250)(251) A 2015 study on climate change affecting wild boars in Europe also reported that milder winters allow for expanded abundance of these mammals.(248)(252). Additionally, feral pigs may migrate seasonally due to food and water availability in California, therefore future projects could incorporate migration patterns and develop risk maps for specific time periods as Lee *et al* (2020) conducted with waterfowl species, although these data are not available yet for most predictors in California. (192)(194)(253)(144)(146)(167)(187) Building real time dynamic risk maps that incorporate remote sensing data, such as satellite information, could be the next step in predicting high-risk disease transmission areas, as built previously for avian influenza by the California Waterfowl tracker.(194) However, tracking birds may be easier than collaring feral pigs.

Chapter 3 combined aspects of Chapters 1 and 2, by determining the prevalence of STEC in feral pigs that reside near domestic swine raised outdoors and predicted possible areas of contact between these two swine populations. Both multilevel logistic models in Chapters 1 and 3 identified outdoor-raised livestock with access to wild areas, such as wetlands or forests, as a significant risk factor for the presence of STEC in samples. One possible pathway for shared pathogens in wild areas may be wildlife contaminating food or water, which are then consumed by livestock. Additionally, Chapter 3 results could be improved upon by using WGS to analyze relatedness between feral pig and domestic pig samples. Although the pathway for pathogen

spillover can be bi-directional and temporality may be unclear, identifying clusters of shared indicator pathogens is an important next step in analyzing disease risks from feral pigs in California and nationwide.(145)(45)

Chapters 2 and 3 analyzed feral pig populations and their risk to farms that raised domestic swine outdoors. In Chapter 3, 45.45% (5/11) of survey respondents observed feral pig presence on their farm, with 36.36% stating that feral pigs had direct contact (i.e., within 100ft or 30.48m) with their domestic pigs in pastures, pens or barns. These results match the risk map from Chapter 2, which overlapped predicted suitable habitat for feral pigs and OPO locations and showed that 49.18% of the 305 OPO identified in California overlapped with suitable feral pig habitat, indicating that spillover of an emerging or transboundary disease is possible, given the correct drivers. We know human or livestock encroaching into tropical forests are drivers for zoonotic diseases such as COVID-19 or Nipah Virus, usually with an intermediate host such as bats.(254) Although emerging zoonotic diseases in many cases originate at the interface of wildlife-livestock-humans, the US is not considered a hot spot for zoonotic disease outbreaks according to Daszak and the EcoHealth Alliance (2017), yet zoonotic pathogen outbreaks can still occur.(255)(256) Possible drivers of disease spillover in the US between feral and domestic pigs raised outdoor include density of animals, shared natural areas between domestic and wildlife and increasing contact between these two growing swine populations.(255)(256)

As the number of DSSF farms continues to grow, continued evaluation of risk factors and agricultural management practices that are unique to these small operations will identify additional risk mitigation strategies and develop extension outreach materials to keep food safe from farm to fork and protect California's agricultural economy. Additionally, as the two parallel trends nationwide of expanding feral pig populations and outdoor-based domestic swine

continues, disease surveillance of feral pigs located near outdoor-raised domestic swine is key in preventing transmission of emerging or reemerging pathogens in the future.

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