THE SUSCEPTIBILITY OF NORTH AMERICAN RACCOONS (*PROCYON LOTOR*) AND STRIPED SKUNKS (*MEPHITIS MEPHITIS*) TO SARS-COV-2 AND THEIR HUSBANDRY IN A CONTAINMENT FACILITY

by

RAQUEL FRANCISCO

(Under the Direction of Sonia M. Hernandez)

ABSTRACT

A sylvatic cycle of SARS-CoV-2 could present multiple opportunities for repeated spillback into human populations and other susceptible wildlife. Based on their taxonomy and natural history, two native North American wildlife species —the striped skunk (*Mephitis mephitis*) and the raccoon (*Procyon lotor*) —represent a high likelihood of susceptibility and ecological opportunity of becoming infected with SARS-CoV-2. In our study, skunks and raccoons were intranasally inoculated or indirectly exposed to SARS-CoV-2. Both species are susceptible to infection; however, the lack of, and low quantity of infectious virus shed by raccoons and skunks, respectively, and lack of cage mate transmission in both species, suggest that neither species are competent reservoirs. However, continued outbreaks in non-domestic species, wild and captive, highlight that additional wildlife research is needed. Herein, we also describe the husbandry and handling techniques developed and effectively utilized for both species in BSL-3Ag to facilitate future research of wildlife in containment.

INDEX WORDS: SARS-CoV-2, COVID-19, Raccoons, Striped Skunks, Mephitidae, Zoonosis, Wildlife, Husbandry, Containment, BSL-3Ag

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DEDICATION

To all those involved in this research,

I am grateful for your camaraderie, enthusiasm, and hard work

To my committee,

I am grateful for your patience, encouragement, and guidance

To friends and family,

I am grateful for your understanding, support, and love

To my corgis,

Rage on

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CHAPTER 1

LITERATURE REVIEW

The Emergence of SARS-CoV-2

In late December 2019, several human patients presented with atypical viral pneumonia of undetermined etiology in Wuhan, Hubei Providence, China. Subsequent virus isolation and phylogenetic work identified a novel coronavirus, Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), as the causative agent. Despite exhaustive efforts to mitigate the virus's escape out of Wuhan, SARS-CoV-2 began to spread across the globe. By late January 2020, the World Health Organization (WHO) declared a Public Health Emergency of International Concern. To date, COVID-19, the associated human disease, has killed over 5.6 million people worldwide and infected close to 355 million (*COVID-19 Dashboard*, 2021).

As with Severe Acute Respiratory Syndrome (SARS), current phylogenetic evidence indicates that SARS-CoV-2 originated from an unknown bat species; several species are known to be natural reservoirs of SARS-like coronaviruses (Li et al., 2020). The most closely related bat coronavirus (BatCoV) RaTG13, was isolated from a horseshoe bat (*Rhinolophus affinis*) in the Yunnan Province and found to form a lineage that included SARS-CoV-2, distinct from other SARS-related coronaviruses (Zhou et al., 2020). Spillover from animals to humans at a wet market in Wuhan remains the most plausible explanation for the initial jump of SARS-CoV-2 from bats to humans; however, uncertainty remains regarding the possibility of an intermediate host (Benvenuto et al., 2020). SARS and Middle East Respiratory Syndrome (MERS) are

believed to have had distinct intermediate hosts (i.e., masked palm civets and dromedary camels, respectively), yet viruses, such as Nipah Virus, are known to spread directly from bats to humans. However, it remains unlikely that SARS-CoV-2 was transmitted directly from bats to humans due to a) the historic need for a mammalian intermediate host among zoonotic betacoronaviruses, b) bats were not found or sold at the Wuhan Huanan Seafood Wholesale Market at the time of the virus's emergence, and c) most bat species were hibernating at the time of the index case (Harapan et al., 2020; Lu et al., 2020). Current theories on the intermediate host of SARS-CoV-2 range from Mayan pangolins to rodents to snakes (Xiao et al., 2020). The possibility of multiple intermediate hosts has also been discussed (S. Yuan et al., 2020; J. Zhao et al., 2020).

Once SARS-CoV-2 had established community spread within Wuhan, it became difficult to extirpate due to the novelty of the virus, yielding numerous unknowns and obstacles, such as incubation and shedding periods, lack of streamlined testing, undefined clinical manifestations, and absence of effective treatments and vaccines. Despite global efforts, the virus began to emerge in highly traveled cities where it then spread to local populations. This was not unsurprising due to original estimates placing the basic reproductive rate (R_O) of this virus between 2.5 and 3; an additional study calculated the median R_O to be 5.9 in the preliminary stages of the pandemic within the United States, while rising as high as 6.4 in other countries (Ke et al., 2021; Pan et al., 2021).

Roughly one year after the initial outbreak of COVID-19, several successful vaccines were developed against the wildtype strain of SARS-CoV-2. As time has progressed, millions of

humans have become infected with the virus thus resulting in numerous mutations and creating multiple variants. Several of the variants that have arisen are considered variants of concern (VOC) due to the locations of aforementioned mutations resulting in possible increased virulence, decreased detectability with diagnostic tests, increased infectivity, and/or vaccine escape (Dubey et al., 2022). Now as many countries enter the second year of the COVID-19 pandemic restrictions, VOCs continue to challenge the hope of resolving the pandemic quickly. While the specifics of how SARS-CoV-2 emerged as a human infectious disease are still debated, it is well accepted that wildlife, environmental pressure, and human health have been integral to the development of this pandemic.

An Introduction to Coronaviruses

Coronaviridae is a well-known family of viruses composed of two subfamilies, *Coronaviridae* and *Torovirinae*. Viruses within this family are composed of positive-sense, single stranded RNA (+ssRNA). They infect a wide range of hosts that extend across several taxa; however, they are most known for their ability to cause disease in mammals. The subfamily Coronavirinae has gained infamy within recent decades and is composed of four distinct genera based on different genetic and serologic properties. Alpha- and Betacoronaviruses, which tend to have mammalian hosts and are

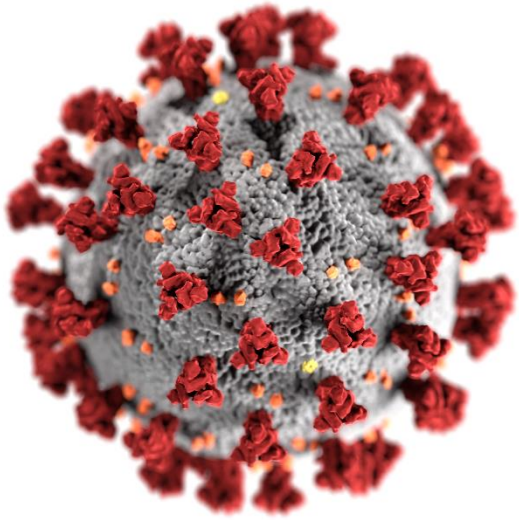


Figure 1.1 An illustration of SARS-CoV-2 molecular structure provided by CDC/ Alissa Eckert, MSMI; Dan Higgins, MAMS. The Characteristic spike proteins are depicted in red.

thought to have originated in bats. Betacoronaviruses are composed of Groups A, B, C, and D; groups C and D contain zoonotic coronaviruses that have spilled over into human populations. The remaining two genera, Gamma- and Deltacoronaviruses, are thought to have originated in and remained in avian hosts (MacLachlan & Dubovi, 2017).

Coronaviruses are well-known for their distinct shape defined by a spike glycoprotein, which is responsible for binding and entry into cells. This approximately 20um protein structure creates a "crown-like" appearance on the exterior of the cylindrical virion. Betacoronaviruses can also have a second set of smaller spikes that can be approximately 5um long. When natural infections of coronavirus occur, neutralizing antibodies have been found to readily adhere to the virion's glycoprotein "spike" (MacLachlan & Dubovi, 2017). Thus, this distinctive spike feature has been used as a primary immunological target on SARS-CoV-2 vaccine trials (Baden et al., 2021; Ledford, 2020; Polack et al., 2020). Having proved efficacious, even against most new variants, the spike protein is the target of most commercially available vaccines (Creech et al., 2021; Liu et al., 2021).

The replication of coronaviruses is complex and often imperfect. RNA viruses are known to have high mutation rates due to decreased proofreading. It is estimated that coronaviruses have one mutation per 3,000 nucleotides during replication, which is equivalent to approximately 10 mutations within the entire genome of each virion. Both the high mutation rates and the virus's predilection toward genetic recombination with coinfections are thought to lead to the immense genetic diversity within this subfamily (MacLachlan & Dubovi, 2017).

Currently, a total of seven coronaviruses, two alphacoronaviruses and five betacoronaviruses, are known to infect humans. The most virulent coronaviruses in humans are the betacoronaviruses: SARS, MERS, and most recently SARS-CoV-2, all of which have been introduced via spillover from wildlife, where they are known to cause no serious disease within their host species (Liya et al., 2020). Coronaviruses also vary in tissue tropism due to their receptor preference. Human coronavirus (HCoV) NL63, SARS, and SARS-CoV-2 utilize angiotensin converting enzyme 2 (ACE-2). ACE-2 receptors are found primarily in both respiratory and gastrointestinal tissues, often resulting in respiratory and gastrointestinal disease upon infection (Ni et al., 2020). In fact, the molecular structure of ACE-2 receptors has recently been studied across a wide variety of species in attempt to predict susceptibility to SARS-CoV-2, thus guiding animal oriented research and conservation efforts (Alexander et al., 2020; Damas et al., 2020).

Coronaviruses are often used as an example of a virus that has the capability of "species-jumping" along with henipaviruses, hantaviruses, filoviruses, and flaviviruses. While MERS and SARS led to isolated epidemics within their respective geographic regions of origin, SARS-CoV-2 has spread despite variable control measures across many regions of the globe, resulting in the worst pandemic of the century. Despite these two previous spillover events (i.e., MERS and SARS), this viral family's determinants for host range specificity and interspecies transmission remain mostly undetermined (MacLachlan & Dubovi, 2017).

A One Health Renaissance

The COVID-19 pandemic has demonstrated that single host-microbe interactions can no longer be examined in isolation. Anthropogenic environmental factors and socio-economic systems

(e.g., habitat destruction, live animal trade, international movement of people and animals, etc.) have drastically influenced host-pathogen dynamics by creating opportunities for pathogens to colonize novel hosts (Åsjö & Kruse, 2006; Estrada-Peña et al., 2014; Keusch et al., 2009; Nava et al., 2017). The One Health approach recognizes the "complex interrelationship between animals, humans and the environment" and encourages collaborative efforts to improve the health of people and animals, including wildlife (Henley, 2020; Lebov et al., 2017; Messmer, 2020). As the human-animal-environment interface expands with humanity's increasing need for resources, the risk of infectious disease emergence will also increase (Hassell et al., 2017; Keusch et al., 2009; Magouras et al., 2020).

Emerging zoonotic diseases (EZD), such as SARS-CoV-2, are characterized by their recent detection or reemergence in a population (Morse, 1995). To begin to understand all facets of a spillover event that resulted in an emergent zoonotic disease, knowledge of the environmental drivers (i.e., climate change, deforestation, agriculture, etc.), human drivers (socioeconomic pressures, cultural practices, etc.), and animal-associated drivers (hunting, ecology, habitat loss, etc.) are needed. For this reason, multisectoral collaborations are essential to understand and mitigate EZDs, and should include governments, local agencies, academia, international partners (i.e., WHO and World Organization for Animal Health (OIE)), and the private sector (Karesh et al., 2012). In a 2009 publication by the United States National Research Council Committee on Achieving Sustainable Global Capacity for Surveillance and Response to Emerging Diseases of Zoonotic Origin, multisectoral collaborative surveillance of humans, livestock, and wildlife were defined as integral aspects of global surveillance and response to emerging zoonotic diseases (Keusch et al., 2009). Moreover, targeted research into the dynamics of these zoonotic pathogens

within their wildlife reservoirs would better inform collaborative One Health efforts to mitigate spillover (Smith et al., 2005).

The COVID-19 pandemic has demanded a One Heath approach across a global platform. Wildlife health assessments, pathogen surveillance, and experimental trials are intrinsic components of the One Health paradigm and have been neglected until recent decades (Cunningham et al., 2017). In a past foreboding quote, the previously mentioned National Research Council stated, "SARS is a good example of such [impromptu] efforts in wildlife: There has been significant research interest in the wildlife origins of SARS in China, yet to date there are no coordinated integrated disease surveillance programs for SARS or other pathogens in wildlife. The lack of human SARS cases since 2003 is one factor for waning interest and loss of commitment to conduct ongoing SARS coronavirus surveillance in wildlife reservoirs" (Keusch et al., 2009). With the COVID-19 pandemic currently causing disease in millions of humans and ravaging the global economy, this seems like a large folly; however, there is little coordinated global EZD surveillance that involves active wildlife sampling (Morse et al., 2012). A One Health approach to SARS-CoV-2 could provide a new ecological context to host-microbe interactions and be utilized to identify potential new routes of exposure and transmission, resulting in novel approaches to preventing and reducing the threat of future EZD outbreaks (El Zowalaty & Järhult, 2020).

Wildlife and its role in the COVID-19 Pandemic

As wildlife habitat continues to degrade, humans and livestock come into increasing contact with free-roaming wildlife. This expanding human-wildlife interface will increase the risk for novel

zoonotic diseases to emerge. Over 70% of the EZDs are known to have a wildlife origin (Jones et al., 2008). RNA viruses comprise 37% of emerging infectious diseases; such pathogens are particularly concerning because of their potential to readily adapt to new hosts given their high mutation rates (Jones et al., 2008; Woolhouse & Gowtage-Sequeria, 2005). Bats are widespread and ecologically vital, and are proven reservoirs of numerous past emergent zoonotic RNA viruses including Lyssaviruses (Warrell & Warrell, 2004), Henipaviruses (Luby et al., 2009; Singh et al., 2019), Ebolaviruses (Leroy et al., 2005), Marburgviruses (Towner et al., 2009), MERS, SARS, and now SARS-CoV-2 (S. K. P. Lau et al., 2005; Li et al., 2020). Despite current and future efforts to reduce spillover (i.e., reducing anthropogenic activity in areas with rich wildlife diversity), humans will continue to engage in practices that adversely affect wildlife health (e.g., wildlife trade, consumption of bushmeat, deforestation, agriculture), creating opportunities for novel endemic bat viruses to emerge in human populations, particularly coronaviruses (Jones et al., 2008).

Given the epidemiology of previous coronavirus outbreaks, the susceptibility of various animal species is a significant factor. Unlike MERS, for which the dromedary camel (*Camelus dromedaries)* is a well-accepted intermediate host, research remains ongoing on determining the identity of a potential intermediate host for both SARS and SARS-CoV-2 (De Wit & Munster, 2013; Susanna K.P. Lau et al., 2017). The current pandemic is mainly thought to have emerged in humans at Wuhan Huanan Seafood Wholesale Market in China with the assistance of an animal intermediate host, which remains unknown (Lam et al., 2020; Xiao et al., 2020; L. Yuan et al., 2020). Similarly, the 2003 SARS epidemic was also thought to have originated in a wet market that supported large quantities of live animal trade. Masked palm civets (*Paguma*

larvata) are often thought of as the intermediate hosts for SARS due to investigative work done at the market in attempt to identify the source of original spillover. The results of this investigation established that three palm civets were PCR-positive for a SARS-CoV-like isolate on nasal and rectal swabs, however one raccoon dog (*Nyctereutes procyonoides*) was also positive on rectal swab. The animals previously listed, in additional to one Chinese ferret badger (*Melogale moschata*), displayed neutralizing antibodies to SARS, suggesting all animals had been previously exposed and infected with SARS (Guan et al., 2003).

Subsequent experimental studies revealed that palm civets were susceptible to SARS-associated disease, reinforcing the possibility that they could have served as an amplifying intermediate host (Freuling et al., 2020; Wang & Eaton, 2007; Wu et al., 2005). However, neither raccoon dogs nor Chinese ferret badgers were evaluated via experimental trials for susceptibility to SARS, leaving the possibility of additional intermediate hosts unanswered. Moreover, the result of this study fortified the belief that wildlife susceptible to SARS-CoV-like viruses living or in close contact with humans could present opportunity for spillover.

Although professionals in the conventional medical field were largely surprised by the emergence of SARS and SARS-CoV-2, wildlife disease researchers and veterinary virologists had a pre-existing understanding of the potential for increased virulence of coronaviruses, as well as for emergence of novel strains from unknown reservoirs (Knobler et al., 2004; Saif, 2004). SARS-CoV-2, like SARS-CoV, is suspected to have a broad host range (Zhang et al., 2020). As SARS-CoV-2 continues to circulate worldwide, the pressing need to determine species specific susceptibility to predict and potentially prevent spillback into domestic animals and native

wildlife from humans has become a priority. This has spurred surveillance and experimental infection trials on numerous species, most of which are depicted in Figure 2 (Berhane et al., 2020; Bosco-Lauth et al., 2020, 2021; Griffin et al., 2021; Y.-I. Kim et al., 2020; Mykytyn et al., 2021; Osterrieder et al., 2020; Palmer et al., 2021; Pickering et al., 2021; Schlottau et al., 2020; Shi et al., 2020; Shuai et al., 2021; Ulrich et al., 2020, 2021; Wernike et al., 2020; Xu et al., 2020; Y. Zhao et al., 2020).

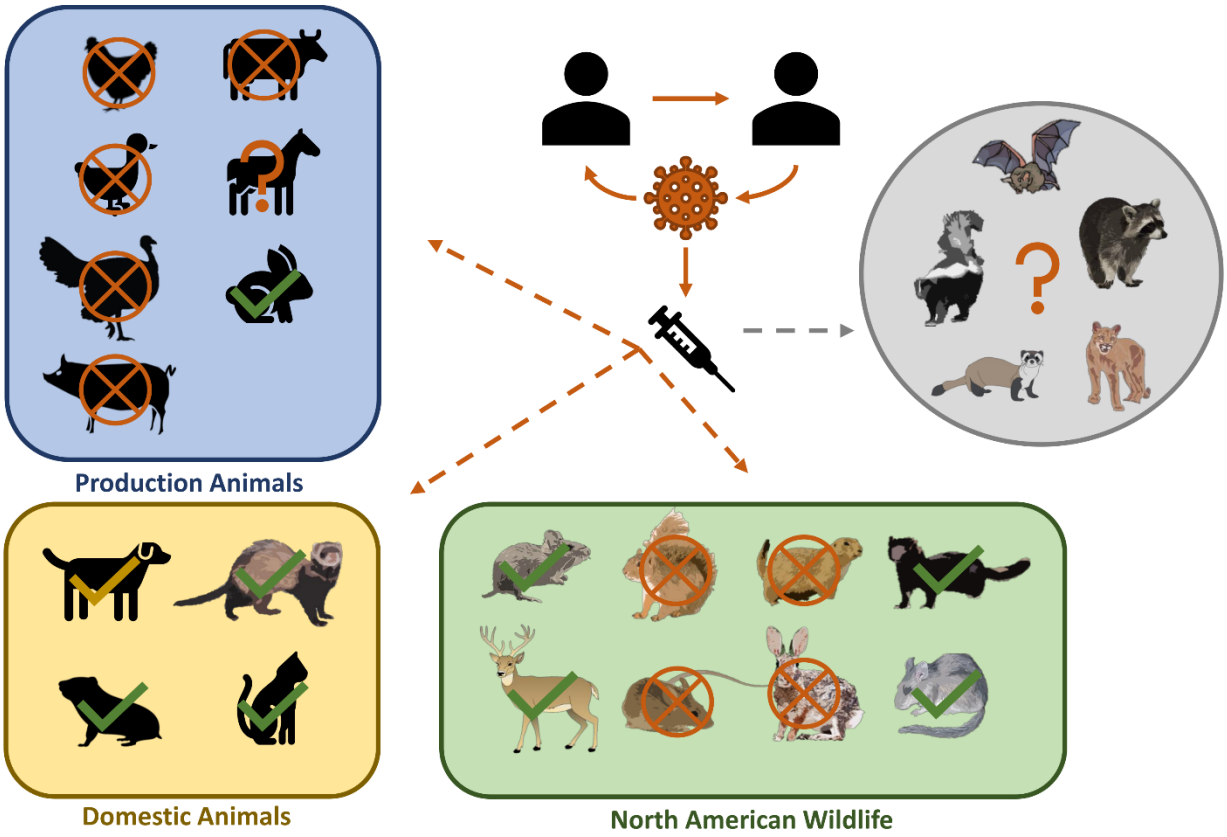


Figure 1.2 Animal species that are confirmed to be susceptible to SARS-CoV-2 via experimental infection trials are depicted above by green check marks. The denoted species were able to contract, shed, and seroconvert to SARS-CoV-2 after challenge; the yellow check mark denotes species that did not shed but seroconverted. The red crosses denote species that are not susceptible to SARS-CoV-2 infection, and thus are unlikely to be reservoirs. The red question mark denotes species of concern that have yet to be challenged in a laboratory setting.

Prior to the COVID-19 outbreak, several species were known to become infected with and shed SARS coronavirus (Gong & Bao, 2018). Many of these same species also share susceptibility to SARS-CoV-2, most notably, domestic cats, non-human primates (NHP), and domestic ferrets (Mullick et al., 2020; Pandey et al., 2020). Shortly after susceptibility trials to SAR-CoV-2 were performed in conventional laboratory species (e.g., mice, ferrets, Syrian hamsters), evaluation of the susceptibility of less conventional species was initiated, not only for use as laboratory models of human disease, but also due to their ecological (e.g., virus transmission and maintenance) implications. This work has revealed several wildlife species that are particularly susceptible, (e.g., Chinese tree shrews (*Tupaia belangeri chinensis*)) that became infected and shed SARS-CoV-2 after direct nasal inoculation (Y. Zhao et al., 2020). While this species was not determined to be a useful animal model, it is now theorized to be an adequate intermediate host. Raccoon dogs, once considered an incidental host for SARS, were also evaluated as a potential reservoir for SARS-CoV-2. Direct nasal inoculation of raccoon dogs with SARS-CoV-2 demonstrated that this species is susceptible to infection, sheds virus readily, and transmits virus to contact animals while showing little to no signs of clinical disease (Freuling et al., 2020). Even after several Asian wildlife species were evaluated for susceptibility to SARS-CoV-2 infection and disease, the status of countless theoretically susceptible wildlife species remains undetermined.

To date, few infection trials have been performed on North American wildlife. Fundamental infection trials were conducted early in the pandemic on both a captive population of deer mice (*Peromyscus maniculatus*) and a captive population of white-tailed deer (*Odocoileus virginianus*). The deer mice were susceptible to SARS-CoV-2 infection following direct

intranasal inoculation. Unlike some animal models (e.g., cats, NHP, ferrets), they showed little to no signs of clinical disease, despite shedding virus readily. The deer mice were also capable of transmitting SARS-CoV-2 to naïve conspecifics through direct contact. This species' lack of severe clinical disease, and ability to shed and transmit virus to conspecifics via direct contact, suggests they are a potential reservoir host of SARS-CoV-2 (Griffin et al., 2021). White-tailed deer (WTD) fawns, similar to deer mice, shed and transmitted virus to conspecifics via direct contact while showing little to no clinical disease after inoculation with SARS-CoV-2; thus, in theory, they also have the ability to be a reservoir host of SARS-CoV-2 (Palmer et al., 2021).

Several articles have followed Palmer et. al.'s (2021) work in hopes of understanding infection dynamics of SARS-CoV-2 in WTD. An additional study experimentally infected WTD fawns then placed the fawns with direct contact fawns to establish when deer-to-deer transmission occurred over time (Martins et al., 2021). Martins et. al. (2021) was able to establish that effective deer-to-deer transmission took place on day post inoculation (dpi) 3, however no direct contact fawns become infected that were introduced past dpi 6. Tissue trophism and infection dynamics was also explored in adult WTD after inoculation (Martins et al., 2021). Another prominent infection trial in WTD found that does can transmit SARS-CoV-2 vertically (Cool et al., 2021). Cool et. al. (2021) also evaluated cytopathic effect (CPE) of SARS-CoV-2 on both Elk (*Cervus canadensis*), Mule deer (*Odocoileus hemionus*), and WTD primary lung cell culture. While elk (subspecies not identified) lung cells do not appear to allow for ancestral virus replication, Mule deer lung cells appear to sustain replication longer than WTD lung cells. Cool et. al. (2021) also found that the more infectious VOC B.1.1.7 outcompetes the ancestral virus in WTD.

With only a few native species evaluated for susceptibility to SARS-CoV-2, large knowledge gaps remain. These susceptibility trials on wildlife are imperative for wildlife health assessments given their field applications. While the outcomes of experimental infection trials may not translate directly into naturally acquired infections *in situ* (i.e., trials may be unable to account for variable demographics, seasons, co-infections, and other stochastic effects), they allow for fact-based conjectures to be made about the effects of natural infections on populations. Wildlife species that are highly adapted to anthropogenic environments and are susceptible to SARS-CoV-2 infection and readily shed virus in an artificial setting should be targeted for surveillance and management in the field in hopes of mitigating spillback. For example, the susceptibility trial in white-tailed deer alerted shareholders that further wildlife surveillance actions were likely needed. In fact, a recent seroprevalence study conducted by the United States Department of Agriculture (USDA), showed that 40% of white-tailed deer in select northeastern states had neutralizing antibodies to SARS-CoV-2, suggesting that these populations experience some level of virus infection (Chandler et al., 2021). Even more studies have followed confirming multiple cases of suspected spillback from humans to both captive and free-roaming WTD (Hale et al., 2021; Kuchipudi et al., 2021; Palermo et al., 2021; Roundy et al., 2021). However, in the case of these deer, as well as most wildlife faced with zooanthroponosis, epidemiology of SARS-CoV-2 within populations remains unknown.

Unintended Consequences: Spillback

Concerns of spillback of SARS-Cov-2 from humans to other animal species were first confirmed with reports of infected domestic cats in Asia between January and March of 2020 (Deng et al., 2020; Zhang et al., 2020). In late April 2020, exotic felids also demonstrated susceptibility to

infection and disease, when tigers and lions at the Bronx Zoo in New York, USA tested positive for SARS-CoV-2 after presumably becoming infected by their caretaker (McAloose et al., 2020). Soon after, numerous cases of domestic dogs and cats becoming infected with SARS-CoV-2 within the USA were reported by the USDA (U.S. Department of Agriculture, 2021). Also, in April 2020, SARS-CoV-2 caused respiratory disease and increased mortality in farmed mink (*Mustela vison*) in several European countries in which workers also tested positive for the virus. It is now accepted that mink became infected from their caretakers (Oreshkova et al., 2020; U.S. Department of Agriculture, 2020b). Mink, like ferrets, display clinical disease upon infection, often showing signs of both respiratory and gastrointestinal disease. Dutch authorities state that there was suspected transmission from mink to human caretakers (e.g., transmission of virus from infected human to uninfected animal then from infected animal to uninfected human), which is the first suggestion of spillback from an intermediate host unrelated to the Wuhan wet markets (Government of the Netherlands, 2020). Shortly after, this claim was confirmed using sequencing and phylogenetic evidence (Munnink et al., 2021). Since this Dutch report, other European countries have reported COVID-19 in farmed mink, including Lithuania and Spain (Pomorska-Mól et al., 2021; U.S. Department of Agriculture, 2020a).

Outbreaks of SARS-CoV-2 continue to affect fur farms globally and have led to the culling of hundreds of thousands of mink. In response to these outbreaks, the USDA released the "Interim SARS-CoV-2 Guidance and Recommendations for Farmed Mink and Other Mustelids", which aimed to increase biosafety measures, including use of personal protective equipment (PPE), and daily animal health screenings of employees (U.S. Department of Agriculture & U.S. Centers for Disease Control and Prevention, 2020). In spite of these guidelines, fur farms across the USA

(e.g., Utah, Oregon, Michigan, and Wisconsin) continue to report SARS-CoV-2 in mink (U.S. Department of Agriculture, 2021). In December 2020, USDA announced a call for licensing and permitting applications for vaccines for SARS-CoV-2 in captive mink in an attempt to control SARS-CoV-2 spread among farmed individuals (Rippke, 2020). Until an efficacious vaccine is implemented to manage SARS-CoV-2 infection in production mink, facilities are forced to cull affected animals. In a recent incident, an Oregon fur production facility purportedly had several SARS-CoV-2 positive individual mink escape their quarantine facility. A total of 3 mink were trapped near the facility and all were presumed to have recently escaped from the quarantine facility based on adequate nutritional condition. Two of trapped the mink tested positive for SARS-CoV-2 (Oregon Department of Agriculture, 2021). Mink, and numerous other mustelids including American martins (*Martes americana*), fishers (*Martes pennanti*), ermines (*Mustela erminea*), long-tailed weasels (*Mustela frenata*), and wolverines (*Gulo gulo*), are native and endemic to Oregon and much of the Pacific Northwest of America. Incidents like this demonstrate how easily SARS-CoV-2 is introduced into naïve (i.e., presumed susceptible) wild populations.

Unfortunately, the discovery of seropositive WTD in multiple states in the Gulf Coast, Midwest, Northeast, and Southeast United States has brought the reality of a possible North American wildlife host for SARS-CoV-2 into center stage. Thus far, virus RNA isolated from free-roaming WTD suggest that deer are becoming infected due to repeated spillback events, however deer-to-deer transmission is heavily implicated in the virus's maintenance in these populations (Kuchipudi et al., 2021). White-tailed deer's possible role in the maintenance of SARS-CoV-2 only re-enforced the importance to understand the susceptibility of additional North American

wildlife, as SARS-CoV-2 is likely to become endemic in humans thus continue to spill back into suspectable host species.

North American Species of Concern

As SARS-CoV-2 made its way into and across North America, introduction, and establishment of the virus into native wildlife became a major concern. In April 2020, the Association of Fish and Wildlife Agencies (AFWA) recommended that state natural resource agencies discontinue bat research and rehabilitation to address concerns of reverse zoonosis into North American bat species (Association of Fish & Wildlife Agencies, 2020), thus protecting declining populations that are struggling against another introduced exotic pathogen (Olival et al., 2020). There is concern for other highly managed endangered species, such as the Black-footed ferret (*Mustela nigripes*), that are taxonomically closely related to hosts (i.e., ferrets, mink) known to be susceptible to coronaviruses (Alexander et al., 2020; Richard et al., 2020; Schlottau et al., 2020).

Unlike the cautious approach demonstrated with North American bats, a captive population of over 100 Black-footed ferrets housed at the National Black-Footed Ferret Conservation Center (NBFFCC) in Larimer County, Colorado received an experimental vaccine produced for large scale use in the mink fur industry in the spring of 2020, despite the lack of clinical data demonstrating susceptibility to SARS-CoV-2. Unlike human mRNA vaccines produced by Pfizer and Moderna, the experimental vaccine developed by Sinovac Life Sciences, contained inactivated virus and while unchallenged, appears to provide adequate immunity in Black-footed ferrets (McReynolds, 2020). Due to the lack of knowledge of SARS-CoV-2 in North American wildlife, various approaches in management of wildlife, from avoidance to active surveillance,

have been enacted during the COVID-19 pandemic. Ideally, controlled experiments that classified the susceptibility in native wildlife species would help better inform management decisions and direct resources appropriately (e.g., the distribution of field equipment, manpower, and laboratory supplies, the extent of PPE needed in the field).

Felidae and Musteloidea (Mustelidae, Mephitidae, and Procyonidae) are two families of North American native terrestrial wildlife that likely are susceptible to SARS-CoV-2 infection and disease (Becker et al., 2020; Benvenuto et al., 2020; Li et al., 2020; Liya et al., 2020). In fact, ferrets and now mink (*Mustela* spp. (Shuai et al., 2021)), which are closely related to native Musteloidea, represent well-established animal models for coronavirus infections (Chu et al., 2008; Martina et al., 2003; ter Meulen et al., 2004; Van Den Brand et al., 2008; Weingartl et al., 2004) and have been shown to be highly susceptible to SARS-CoV-2 (Y. Il Kim et al., 2020; Richard et al., 2020; Shi et al., 2020). Even though Mustelids remain an ecologically relevant species due to their high probability of becoming infected with and shedding SARS-CoV-2, and displaying clinical disease, no susceptibility trials have been performed on non-production species in this family. Other families with demonstrated susceptibility (e.g., free-living members of the Felidae, barring feral cats) have fewer ecological opportunities for spillback. However, recent reports of high prevalence of SARS-CoV-2 infection in WTD, the largest portion of a Florida panther's (*Puma concolor coryi*) diet in respect to biomass may increase the exposure of these endangered big cats (U.S. Fish and Wildlife Service, 2008). In contrast, prolific urban wildlife species, such as striped skunks and raccoons, have strong taxonomic relationships to established susceptible species, are abundant, and regularly come in contact with humans and human-associated waste material.

Striped skunks (*Mephitis,* Mephitidae) and Raccoons (*Procyon lotor,* Procyonidae) belong to the super family musteloidea; they have established populations throughout most of the continental USA and are opportunistic, omnivorous generalists (Glatston, 1994; Zeveloff, 2002). While often trapped or hunted in a natural setting for fur, both species are well adapted to urban and rural areas. Specifically, raccoon densities increase significantly with the availability of food sources in urban/suburban (e.g., trash cans and feral cat feeding stations) and agricultural areas (e.g., crop fields) (Beasley et al., 2007; Prange et al., 2003, 2004). Both species have become habituated to food and shelter near human homes, resulting in frequent interactions with domestic pets and humans. As with other Musteloidea, skunks and raccoons are notorious reservoirs of viruses that have potentially high impacts on other wildlife and humans (e.g., rabies, canine distemper) (Charleton, K.M., Webster, W.A., & Casey, 1991; Williams & Barker, 2001). Raccoons have interconnected and expansive social networks, particularly during the winter and mating seasons, increasing the risk for pathogen transmission (Hirsch et al., 2013; Prange et al., 2003; Reynolds et al., 2015). Furthermore, raccoons are susceptible to viral pathogens of both canids and felids, often developing severe clinical disease from viral pathogens that routinely cause explosive and cyclical epizootics, including rabies virus, feline and canine variants of carnivore protoparvovirus, and canine distemper (Allison et al., 2012, 2013).

In several states, both of these species continue to be sold as pets and commonly present to wildlife rehabilitation centers for treatment from domestic animal attacks, vehicular collisions, infectious diseases, and other causes. In fact, ~12,000 raccoons are rehabilitated and released throughout North America per year; this high number likely is in part due to their charismatic

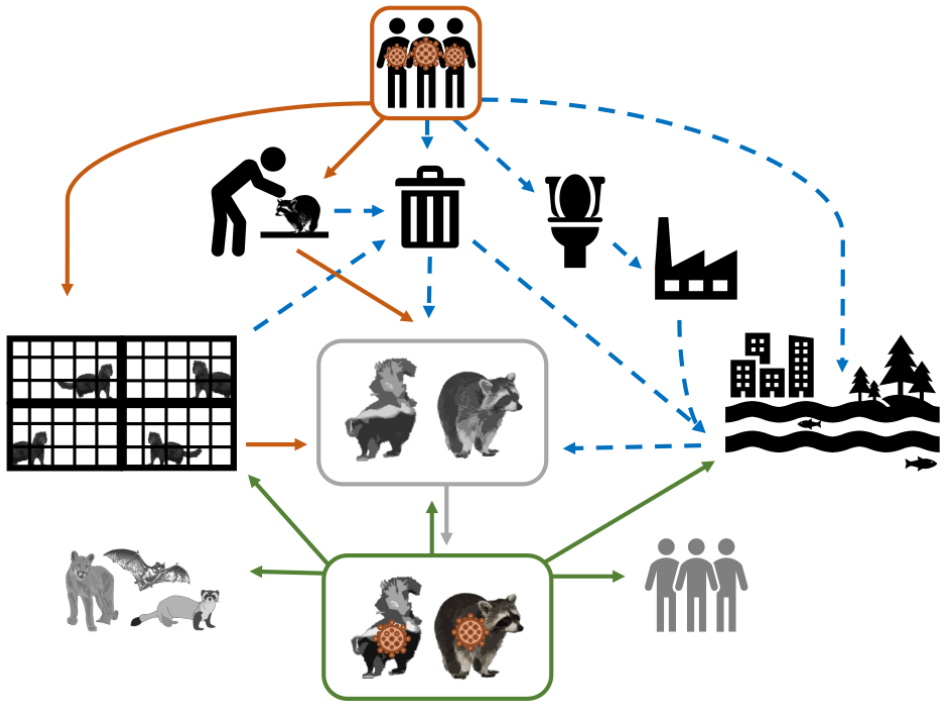
nature (Rosatte, 2000); E. Miller pers comm). Owing to their opportunistic nature, these species are commonly, directly and indirectly, exposed to humans and their waste. As reiterated in Figure 3, there are many avenues by which either species is theoretically exposed to SARS-CoV-2 and in turn act as a zoonotic reservoir if susceptible (Franklin & Bevins, 2020).

Rational and Significance

To date, a small amount of literature can be found on experimental infections of North American wildlife; in particular, native mesomammals. Thus far, only one preliminary preprint is available that evaluates the susceptibility of some peridomestic wildlife species where a small sample size of raccoons (n=3) and striped skunks (n=6) were inoculated (Bosco-Lauth et al., 2021). We hope to broaden the knowledge base of SARS-CoV-2 susceptibility and infection outcomes in wildlife by experimentally infecting a larger sample size of two native North American species, striped skunks (n=16) and raccoons (n=16). Both species represent a high likelihood of susceptibility and ecological opportunity for spillback, with SARS-CoV-2. Given the taxonomic relationships between skunks and raccoons and other susceptible species (e.g., ferrets and mink), as well as their abundance in both the natural and human-altered environments, they are ideal candidates for the first SARS-CoV-2 susceptibility study in North American Musteloidea.

Figure 1.3 Conceptual model of proposed mechanisms of SARS-CoV-2 transmission from infected humans (direct transmission = solid orange arrows; and indirect transmission = dashed blue arrows) to susceptible wildlife (represented in grayscale). As depicted, SARS-CoV-2 shed by humans can be directly transmitted through activities that require handling and close contact (e.g., research and wildlife rehabilitation), or commercial operations (e.g., fur farms); however,

virus shed by humans could make its way into the environment via garbage (i.e., medical and household waste) and sewage. The solid gray arrow represents the establishment of SARS-CoV-2 in a wildlife species. The hypothesized spillback from this SARS-CoV-2 wildlife reservoir to susceptible human populations and other wildlife species is demonstrated by the solid green arrows.



If either of these two target species is susceptible to SARS-CoV-2, the potential eco-epidemiological implications are vast. A competent, highly urbanized, native wildlife reservoir of SARS-CoV-2 would present multiple opportunities for repeated spillback into human populations via both direct and indirect contact. Humans are often directly exposed to raccoons and skunks via rehabilitation centers, pest removal services, and the hunting and trapping of these animals. Indirect exposure of skunks and raccoons to humans can also occur in a variety of ways, from animal waste on residential property that contaminates food or highly trafficked

surfaces to exposure of a pet (i.e., cat) that later exposes its owners or acts as a fomite. Other factors, such as increased visitation to parks and urban green spaces, further increase the human-urban wildlife interface and must also be considered (Geng et al., 2021).

There are also conservation implications if a ubiquitous and common North American species, like raccoons or skunks, become reservoirs for SARS-CoV-2. For example, this could pose a threat to highly endangered species such as the Black-footed ferret (BFF), which was recently decimated in the 1980s due to introduced canine distemper virus (Thorne & Williams, 1988), and the Florida panther, which recently suffered a severe genetic bottleneck and population decline due to anthropogenic activities (Johnson et al., 2010). BFF are intensively managed native mustelids that reside in the Mountain West and Southwest regions of the USA that struggle to maintain wild populations. Due to the susceptibility of other mustelid subspecies (i.e., ferrets and mink), it is likely that BFF are susceptible to SARS-CoV-2 associated disease if exposed to a shedding animal.

A variety of felid species have proven to be competent hosts for SARS-CoV-2. Throughout the pandemic, numerous domestic cats, as well as a small number of large exotic felids, have tested positive for SARS-CoV-2 and exhibited clinical disease (McAloose et al., 2020; U.S. Department of Agriculture, 2021). Raccoons comprise a substantial proportion of the Florida panther's diet; thus, if SARS-CoV-2 circulates among native raccoon populations, panthers would be expected to be regularly exposed to SARS-CoV-2, likely resulting in infection and disease (Facemire et al., 1995; U.S. Fish and Wildlife Service, 2008). Unfortunately, much of

this currently remains conjecture because the susceptibility of a large number of North American wildlife species to infection and disease remains unknown.

The COVID-19 pandemic has continued to evolve since the regional and subsequent intercontinental spread of SARS-CoV-2 were first documented in the continental USA in 2020. With total human infections increasing, even with established vaccination campaigns in many countries, it has become increasingly likely that SARS-CoV-2 will become globally endemic (Baker et al., 2021; Lavine et al., 2021). Due to the rapid emergence of knowledge and evolving mitigation strategies in humans concerning SARS-CoV-2, an adaptive strategy to managing native wildlife species is needed. A One Health approach calls for collaborative efforts among federal agencies (i.e., CDC, USFW, and USDA), state agencies, academic institutions (e.g., Southeastern Collaborative Wildlife Disease Study at the University of Georgia College of Veterinary Medicine), private institutions (e.g., fur production facilities), and the public (e.g., hunters, veterinarians, nature enthusiasts) to cultivate these strategies for species of interest. While both academic and federal research groups within the USA continue to research the implications of SARS-CoV-2 in North American wildlife, little information has been published or made accessible. We hope to fill a gap in this knowledge by assessing the susceptibility of striped skunks and raccoons to SARS-CoV-2. Evaluating the results of this study within a One Health context will allow us to better assess the risks of spillback from people to animals and animals to animals, thus impacting the management and surveillance of these species and ultimately, preventing or reducing the threat of future outbreaks.

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CHAPTER 2

EXPERIMENTAL SUSCEPTIBILITY OF NORTH AMERICAN RACCOONS (*PROCYON LOTOR*) AND STRIPED SKUNKS (*MEPHITIS MEPHITIS*) TO SARS-COV-2[1]

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ABSTRACT

Recent spillback events of SARS-CoV-2 from humans to animals has raised concerns about it becoming endemic in wildlife. A sylvatic cycle of SARS-CoV-2 could present multiple opportunities for repeated spillback into human populations and other susceptible wildlife. Based on their taxonomy and natural history, two native North American wildlife species —the striped skunk (*Mephitis mephitis*) and the raccoon (*Procyon lotor)* —represent a high likelihood of susceptibility and ecological opportunity of becoming infected with SARS-CoV-2. Eight skunks and raccoons were each intranasally inoculated with one of two doses of the virus (10^3 PFU and 10^5 PFU) and housed in pairs. To evaluate direct transmission, a naïve animal was added to each inoculated pair 48 hours post-inoculation. Four control animals of each species were handled like the experimental groups. At predetermined intervals, we collected nasal and rectal swabs to quantify virus shed via virus isolation and detect viral RNA via rRT-PCR and blood for serum neutralization. Lastly, animals were euthanized at staggered intervals to describe disease progression through histopathology and immunohistochemistry.

No animals developed clinical disease. All intranasally inoculated animals seroconverted, suggesting both species are susceptible to SARS-CoV-2 infection. The highest titers in skunks and raccoons were 1:128 and 1:64, respectively. Low quantities of virus were isolated from 2/8 inoculated skunks for up to day 5 post-inoculation, however no virus was isolated from inoculated raccoons or direct contacts of either species. Neither species had gross lesions, but recovering mild chronic pneumonia consistent with viral insult was recorded histologically in 5/8 inoculated skunks. Unlike another SARS-CoV-2 infection trial in these species, we detected neutralizing antibodies in inoculated raccoons; thus, future wildlife serologic surveillance results

must be interpreted with caution. Due to the inability to isolate virus from raccoons, the lack of evidence of direct transmission between both species, and low amount of virus shed by skunks, it seems unlikely for SARS-CoV-2 to become established in raccoon and skunk populations and for virus to spillback into humans. Continued outbreaks in non-domestic species, wild and captive, highlight that additional research on the susceptibility of SARS-CoV-2 in wildlife, especially musteloidea, and of conservation concern, is needed.

INTRODUCTION

As severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) continues to circulate on a global scale, the need to identify potential animal reservoirs, especially among wildlife, has become a priority, spurring surveillance and susceptibility trials of numerous species (1–15). Indeed, the COVID-19 pandemic has highlighted the need for a global One Health approach to address its solution (16). Wildlife health assessments, pathogen surveillance, and experimental trials are intrinsic components of this approach which have been neglected until recent decades, but have been integral in understanding the epidemiology of other recent pandemics such as severe acute respiratory syndrome (SARS) and middle eastern respiratory syndrome (MERS) (17–19).

Ongoing spillover events from humans to pets (e.g. dogs and cats), commercial animals (e.g. mink), and captive wildlife (e.g. tigers, gorillas) have raised concerns about the ability of SARS-CoV-2 to become endemic in abundant native wildlife species (20–22). A sylvatic cycle of SARS-CoV-2 could present multiple opportunities for repeated spillback into human populations and susceptible wildlife species. While the role of free-living wildlife in the emergence of

SARS-CoV-2 remains unclear, the susceptibility and potential of a wildlife species as a reservoir could hold substantiable implications, not just for public health, but for the management, research, rehabilitation, and conservation of other susceptible animal species (23). In North America, several members of the Musteloidea (Mustelidae, Mephitidae, and Procyonidae) families are both taxonomically and ecologically relevant and likely have a high probability of becoming exposed to, infected with, and developing clinical disease to SARS-CoV-2 (24–28). In fact, ferrets (*Mustela putorius furo*), close relatives to North American Musteloidea, are well-established animal models for SARS (29–32) and are highly susceptible to SARS-CoV-2 (8–10,33–38) Another close relative, mink (*Neovison vison*), is also highly susceptible to SARS-CoV-2 in experimental inoculations trials, as well as natural infections in commercial farms (5,39–43). Most recently, Asian small-clawed otters (*Aonyx cinereus*) in a zoological institution were infected with SARS-CoV-2 (44). Like ferrets, both mink and otters experienced varying levels of respiratory disease upon infection with SARS-CoV-2. Unlike ferrets, both species were first found to be susceptible after transmission from an infected human caretaker, highlighting the anthropozoonotic potential of this virus (45).

Two Musteloidea, striped skunks (*Mephitis mephitis,* Mephitidae) and raccoons (*Procyon lotor*, Procyonidae), range throughout much of North America, and are abundant, opportunistic, omnivorous generalists (46). Raccoons are also well established in regions of Europe and Asia (47). Both species have become habituated to seek food and shelter near human homes, resulting in frequent interactions with domestic animals, humans, and their waste. Figure 1 depicts several hypothetical pathways from which SAR-CoV-2 can be transmitted from humans, both directly and indirectly, to species that have ample ecological opportunity, such as raccoons and skunks,

justifying their importance as potential reservoirs. Skunks and raccoons are already notorious reservoirs of viruses that have substantial impacts on other wildlife and humans (i.e., rabies virus, canine distemper virus, protoparvoviruses) (48,49). To determine their role in the epidemiology of SARS-CoV-2, this study evaluated the susceptibility to infection, seroconversion, transmission potential between conspecifics, tissue tropism, and pathology associated with SARS-CoV-2 in striped skunks and raccoons.

METHODS

Animals and Husbandry

Sixteen juvenile (~10 week old), equal numbers of both sexes, captive-bred raccoons and skunks were obtained from a commercial, captive breeding animal facility in June and July 2020, respectively. Animals were either housed at the University of Georgia either in a Biosafety Level 2 (BSL-2) facility (control skunks) or in the Animal Health Research Center (AHRC; experimentally inoculated animals of both species and control raccoons) which is a high-security biocontainment facility. All of the experimental infection work was conducted under Biosafety Level 3 (BSL-3) protocols. All procedures involving the handling of animals and the SARS-CoV-2 virus were reviewed and approved by the University of Georgia's IACUC committee (A2020 04-016) and Office of Biosafety (2020 0048).

Animals were housed at ~21˚C and 50% humidity. Both species were fed daily with commercially available omnivore diet (Mazuri® Omnivore Diet, Purina Mills, LLC., USA) and offered water *ad libitum*. The diet was supplemented by various fresh greens and protein items such as boiled eggs. Animals were identified by purposely shaved patches of fur either on the

left, right, or center of their rump. Prior to inoculations, nasal swabs, rectal swabs, and blood samples were collected and tested by microtitration serum neutralization (SN) and virus isolation (VI) to ensure animals were not currently or previously infected with SARS-CoV-2.

Experimental Design

Experimental animals (n=12), excluding the control animals (n=4) who were housed separately, were separated into 2 identical dosing groups with equal sexes per group. Each dose group consisted of four animals housed in pairs in two adjacent stainless-steel wire mesh cages (~1.5x1.5x2m). The high (H) and low (L) dose animals were intranasally inoculated with 10^3 PFU and 10^5 PFU of SARS-CoV-2 (n=4 per dose, per species), respectively. The 10^5 PFU dose has produced infections in ferrets and other species (9,10). The 10^3 PFU dose was used to mimic the amount of virus to which these species may be naturally exposed (e.g., through consuming human garbage or potentially animal-to-animal) and has also resulted in infections and clinical disease in ferrets (31,37). Each animal was identified by a unique combination of numbers and letters that corresponded with their dosage group, their enclosure number, and the side where a section of their fur was shaved (i.e., raccoon H1L equated to high-dose raccoon from group 1 that shaved on the left side). All four experimental dose groups were housed in the same BSL-3 Agriculture (BSL-3Ag) room but were separated by approximately 6 meters and the directional air flow in the room flowed from the low to the high dose group (Supplementary Figure 1). The design of the BSL-3Ag facility does not allow for recirculated air, facilitating 13 to 15 air changes per hour, thus the likelihood of aerosol transmission between each group is negligible. To test for direct contact transmission, a single naive conspecific was introduced to each pair of

directly inoculated animals 48 hours after inoculation. Control animals (n=4) were housed in either a separate BSL-3Ag room (raccoons) or BSL-2 facility (skunks).

Virus and Inoculations

The SARS-CoV-2 isolate used was USA-WA1/2020 which was originally isolated from a middle-aged male in Washington, USA who traveled to Wuhan China in January 2020. Skunks and raccoons were inoculated with 5th passage virus. The virus was grown in vero-E6 cells (American Type Cell Culture [ATCC] Cat# CRL-1586, RRID:CVCL 0574) which were maintained in minimal essential medium (MEM, 5 L deionized water, 48 g of Minimal Essential Media Eagle (Sigma-Aldrich, Co., USA), 11.11 g bicarbonate) supplemented with 50 mL/L of iron fortified calf serum (Sigma-Aldrich, Co.) and 20 mL/L of Antibiotic Antimycotic Solution (10,000 units penicillin, 10 mg streptomycin, 25 µg amphotericin per mL). All cultures and microtitrations were incubated in a 5% CO_2 atmosphere and 37˚C.

For procedures, such as inoculation and venipuncture, raccoons and skunks were anesthetized with a combination of dexmedetomidine (0.04 mg/kg) (Dexdomitor™, Orion Corporation, Finland) and butorphanol (0.2 mg/kg) (Torbugesic™, Zoetis Manufacturing and Research, Spain), intramuscularly (IM), and reversed with atipamezole (0.25 mg/kg) (Revertindine™, Modern Veterinary Therapeutics, Germany) and naloxone (0.02 mg/kg) (Wintac Limited, India) given IM to return them to pre-anesthetic function as rapidly as possible.

The intranasal inoculations were performed on anesthetized animals using a 21-gauge catheter attached to a 1 mL luer slip syringe (BD Syringe, Becton, Dickinson and Company, USA).

Experimental animals that were intranasally inoculated with live virus (n=8) will be referred to as the directly inoculated (DI) animals or groups. A single direct contact (DC) animal was introduced to each pair of DI animals 48 hours post-inoculation to evaluate direct transmission.

Sampling

Animal health status (i.e., mentation, attitude, physical appearance, consumption of food) was evaluated twice daily. All animals were weighed at admission, and additional body weights were recorded for all animals on the days when they were fully anesthetized for venipuncture. Rectal temperatures were collected from all animals when anesthetized for venipuncture, normothermic was considered 37.2 to 39.2˚C (99.0 to 102.5˚F) for both species (50,51). To collect serum, 2 mL of blood was drawn from the jugular vein and added to plain sterile vacutainer tubes (3 mL; Covidien™, USA).

For the collection of nasal and rectal swabs, animals were physically restrained and sedated with 30-45 mg/kg trazodone PO (Cadila Healthcare Ltd., India) suspended in either water or equal parts of ORA-Plus® Oral suspending vehicle and ORA-Sweet® (Perrigo, USA) in a syringe. The blood and swab collection scheme for both species is summarized in Figure 2. To evaluate environmental transmission, swabs of food and water bowls were obtained each sampling period prior to any animals being handled. Swabs and blood samples were also collected for all animals when euthanized.

Nasal swabs were obtained by swabbing both sides of the nasal passage using a single sterile polyester swab (Puritan, USA). Rectal swabs were obtained using sterile cotton swabs (Medline

Industries, Inc., China). All swabs were placed in 1.5 mL cryovials (SealRite®) with 1 mL of Dulbecco's sterile phosphate-buffered saline (dPBS) (Sigma-Aldrich, Co.) for raccoons and 1 mL of sterile virus isolation medium composed of MEM for skunks. The blood samples and swabs were maintained in an insulated container with frozen gel packs until stored, and the whole blood was centrifuged within 1 hour for the collection of serum. All swabs and serum were then stored at -80˚C until processed.

Animals were anesthetized, euthanized, and necropsied at predetermined intervals to maximize the chance of detecting histopathologic changes during the course of infection. All animals were sampled as described above after humane euthanasia. Animals were anesthetized with dexmedetomidine (0.04 mg/kg) (Dexdomitor™), butorphanol (0.2 mg/kg) (Torbugesic™), and ketamine (5 mg/kg) (Zetamine™, OneVet, USA), and euthanized with an intracardiac dose of sodium pentobarbital (0.25 mL/kg) (Euthanasia Solution, Med-Pharmex Inc., USA). All animals were necropsied the day of euthanasia. Raccoons were euthanized on 9 dpi (n=5; two DI from each dose group, and a control), on 11 dpi (n=3; one DC one from each dose group, one control). The remaining experimental and control raccoons were euthanized and necropsied on 17 and 18 dpi, respectively. The experimental infection trials were performed first on raccoons. Due to the unremarkable nature of the raccoon gross necropsies, the necropsy interval was changed for skunk infection trials. Necropsies were performed earlier to capture early and subtle pathologic lesions such that skunks were euthanized on 4 dpi, (n=3; two DI from each dose group, and a control), and on 8 dpi, (n=4; two DI and two DC from each dose group) similar to Schlottau et al. and Freuling et al. (9,52). A control skunk was euthanized on 7 dpi for comparison. The

remaining control and experimental skunks were euthanized and necropsied on 14 and 15 dpi, respectively (Figure 2).

Sample Analysis

Virus isolation and Molecular Testing: Swab samples were placed in individual microcentrifuge tubes containing 1 mL of viral media, vortexed, and then centrifuged at 10,000 rpm for 10 minutes. Supernatant (100 μL) from each tube was inoculated into a separate well on a 12-well plate seeded with 3-to-4-day old Vero E6 cell culture monolayers. The plates were observed daily for cytopathic effect (CPE) for 10 days. If CPE was evident, the cell culture supernatant was collected and tested for the presence of SARS-CoV-2. Viral RNA (vRNA) was extracted from positive samples using the QIAamp Viral RNA Mini Kit (Qiagen Inc.), following the manufacturer's protocol. A validated real-time reverse transcription PCR (rRT-PCR) protocol was used for detection of SARS-CoV-2 (53,54). Reactions were conducted on a Step OnePlus Real-Time PCR System (Applied Biosystems, Inc.).

The same rRT-PCR protocol was used as described above to evaluate tissues and nasal, fecal, and environmental swab samples for the presence of SARS-CoV-2 RNA. A positive rRT-PCR result was defined as the detection of both the N1 and N2 genes. Both the N1 and N2 primer/probe had to have a cycle threshold (Ct) of ≤35 to be considered positive for the presence of SARS-CoV-2 RNA. Samples evaluated that resulted in a Ct of >35 for both probes were considered negative and samples with a Ct of ≤35 for one probe and a Ct of >35 for the other probe were also considered negative as reported in Shriner et al. (41). Viral stock with a titer of 10^5 pfu/200ul was used as a positive control.

Skunk tissues (nasal conchae, tracheobronchial lymph node, tonsil, mid-length trachea, right middle lobe lung, heart, kidney, and jejunum) and select raccoon tissues (tracheobronchial lymph node, tonsil, right middle lobe lung) samples were homogenized with gentleMACS™ C Tubes (Miltenyi Biotec Inc., Germany) using a gentleMACS™ Dissociator (Miltenyi Biotec Inc.). Tubes were then centrifuged at 3,220 rpm for 10 minutes at 22˚C. Then, 100 µL of supernatant from each tube was inoculated into a separate well on a 12-well plate seeded with 3-to-4-day old Vero E6 cell culture monolayers. CPE was determined as discussed above. An additional 140 µL of supernatant from each tube was collected and tested for the presence of SARS-CoV-2 using the extraction protocol and rRT-PCR protocol listed above.

Plaque Assays to Quantify Virus: Cell culture supernatant (200 µL) from samples that were positive for SARS-CoV-2 via VI and rRT-PCR was diluted 10-fold (with the first well containing no dilution) for a series of 5 dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}), inoculated into a 6-well plate previously seeded with 4-day old Vero E6 cell culture monolayers, and incubated at 37°C and 5% CO_2 for 1 hour. Each well was then overlaid with 4 mL of a gum tragacanth overlay solution (equal parts 2% gum tragacanth & 2XMEM, supplemented with 2 mL of Fetal Bovine Serum (FBS), 5 mL of Antibiotic Antimycotic Solution) and allowed to incubate as described above for 7 to 10 days. Once plaques were noted grossly, each cell culture was inactivated with 10% formalin and crystal violet solution and allowed to fix for 24 to 48 hours. Once cells were fixed, SARS-CoV-2 titers ($\log_{10}$ PFU/mL) were evaluated in wells for which more than one plaque was present; no plaques were seen past 10^{-3} dilution on any sample. As previously determined, a ½ log is lost for each freeze thaw cycle, and all vials had been through

2 cycles, presumptively decreasing titers by 1 log (D.G. Mead and E.R. Lafortaine, unpublished data).

Microtitration Serum Neutralization: SARS-CoV-2 neutralizing antibodies were detected and quantified using serum microneutralization. Serum samples were heat-inactivated at 56°C for 30 minutes. Then, samples were 2-fold serially diluted in duplicates from 1:4 to 1:256 and incubated at 37°C and 5% CO_2 with 100 $TCID_{50}$ of the same strain of virus used in the inoculum in 96-well plates for one hour. The wells were then overlaid with 150 µL of Vero E6 cells. The plates were incubated as described above and observed for CPE daily for 7 to 10 days, after which sample neutralization endpoint titers were determined.

Necropsy, Histology, and Immunohistochemistry: All inoculated and control animals were necropsied within 2 hours of euthanasia. Approximately 0.5 cm^3 samples of nasal conchae, tracheobronchial lymph node, tonsil, mid-length trachea, right middle lobe lung, heart, kidney, and jejunum were placed in cryovials and stored at -80°C for subsequent laboratory analyses. Additional samples collected into 10% neutral buffered formalin for histopathologic evaluation included nasal sinus, trachea, left cranial and caudal lung lobes, right cranial and middle lung lobes, bronchus, lymph nodes (tracheobronchial, retropharyngeal, prescapular, and mesenteric), tonsil, tongue, esophagus, duodenum, jejunum, ileum, stomach, large intestine, left lateral liver lobe, gall bladder, pancreas, spleen, heart, kidney, thymus, thyroid gland, adrenal gland, gonad, skeletal muscle (biceps), urinary bladder, bone marrow, cerebrum, cerebellum, brainstem, and eye.

Once fixed, nasal sinus tissues were transferred to 12.5% neutral EDTA solution (250 g EDTA disodium salt (J.T. Baker Inc. USA), 1750 mL distilled water, and 25 g sodium hydroxide) where they were allowed to decalcify for 14 to 21 days. Fixed tissues were routinely processed, embedded in paraffin wax, and 4 μm thick sections were stained with hematoxylin and eosin (HE). Duplicate slides with deep nasal sinus, mid-trachea, left cranial lung lobe, bronchus, tracheobronchial and prescapular lymph nodes, tonsil, and jejunum for all raccoons inoculated with low and high SARS-CoV-2 doses also underwent immunohistochemistry (IHC) for SARS-CoV-2 antigen. These same tissues, in addition to frontal nasal sinus, left caudal lung lobe, right cranial lung lobe, retropharyngeal lymph node, kidney and heart also underwent IHC for all skunks inoculated with low and high doses, as well as the two high dose direct contact skunks. IHC was performed on an automated stainer (IntelliPATH, Biocare Medical, USA). A rabbit polyclonal antibody for SARS-CoV-2 (ThermoFisher, PA141098) at a dilution of 1:100 for 60 minutes was used. Antigen retrieval on tissue sections was achieved using Citrate Solution 10X (BioGenex, Fremont, USA) at a 1:10 dilution 10 for 15 minutes at 110°C. A biotinylated goat anti-rabbit antibody at a 1:100 dilution (Vector Laboratories, USA) was utilized to detect the target, and immunoreaction was visualized using Warp Red Chromogen (Biocare Medical) for 10 minutes and counterstained with hematoxylin. A cell pellet with infected cells was used as a positive control. All histology and immunohistochemistry were performed at the Athens Veterinary Diagnostic Laboratory at the University of Georgia and slides were read blindly by a board-certified veterinary pathologist.

RESULTS

None of the experimental animals of either species developed a fever (e.g., rectal temperature > 39.2˚C), lost weight, changed behavior or displayed any signs of clinical disease throughout the study. Viral shedding was only detected on nasal swabs by virus isolation from two high dose DI skunks (H2R on 3, 4, and 5 dpi and H1L on 1 and 2 dpi) (Table 1). The highest amount shed was 3.3 $\log_{10}$/mL on 4 dpi. Virus was not isolated from any raccoon swabs, raccoon tissues, skunk rectal swabs, or skunk tissues.

All skunk and select raccoon samples were evaluated for the presence of vRNA using rRT-PCR (Ct of ≤35). SARS-CoV-2 RNA was detected in the nasal swabs of 3/8 DI raccoons (H1R, H2L, L2L) and 4/8 DI skunks (H1L, H1R, H2R, L2R); this includes two skunks, H1-L and H2-R, from which virus was isolated as displayed in Table 2. In addition, skunks H1-L and H2-R were the only animals in this study to have Ct values ≤ 28, a threshold that coincides with historic human data for obtaining culturable virus when evaluating the samples for the SARS-CoV-2 N gene via RT-PCR(Supplementary Table 2) (55). Viral RNA was also detected from one high dose DI skunk (H2R) nasal turbinate tissue sample from 8 dpi. No vRNA was detected from skunk or raccoon rectal swabs, skunk environmental swabs, or raccoon tissues. All directly inoculated animals of both species seroconverted—defined by a 4-fold increase in antibody titers—by the end of the study; however, no seroconversion occurred in any direct contact animals. The highest titer was 1:64 in raccoons and 1:128 in skunks, and the earliest seroconversion timepoint was on 9 dpi and 8 dpi, respectively (Supplementary Table 1).

The gross necropsies for all animals were unremarkable. No microscopic lesions or SARS-CoV-2 specific immunohistochemical labeling were evident in tissues from raccoons. The frontal and deep nasal conchae of three DI high dose skunks (H1R, H2R, H2L) and two DI low dose skunks (L1R, L2L) had mildly to moderately increased numbers of widely scattered lymphocytes and plasma cells in the superficial lamina propria vs. DC and control animals. At least one of four examined sections of lung, either the left cranial/caudal or right cranial/middle lung lobes, of DI high dose skunks (H1R, H2R, H1L) and DI low dose skunks (L2R, L2L) had mildly increased numbers of perivascular lymphocytes and plasma cells randomly scattered throughout the interstitium. There was no corresponding immunohistochemical labeling in these nor in any other tissues examined from skunks. Incidentally, all skunks had moderate to severe, diffuse hepatic lipidosis and one DI skunk (L1R) had focal, purulent rhinitis in the frontal nasal conchae. All skunks had robust lymphoid tissue in lymph nodes, spleen, bronchus-associated lymphoid tissue (BALT; lungs), and gastrointestinal-associated lymphoid tissue (GALT; intestine).

DISCUSSION

A One Health approach to understand and mitigate the epidemiology and management of SARS-CoV-2 in a wide range of hosts calls for a collaborative effort between governmental agencies, academic and private institutions, and the public (56). Such efforts must include experimental trials, wildlife health assessments, and pathogen surveillance (57). In terms of the number of human infections and deaths and the currently known animal host range, the COVID-19 pandemic is one of the most important global emerging zoonoses to date, which will take a concerted multidisciplinary approach to manage.

Our findings demonstrate that while striped skunks and raccoons are susceptible to SARS-CoV-2 infection, it is unlikely that either species is likely to be a competent reservoir for SARS-CoV-2 in a natural setting. No animals experienced clinical disease during this study. Raccoons exhibited no pathology, and skunks had mild evidence of subclinical recovering cellular response to viral infection in the nasal conchae and lungs. The lack of virus isolation from raccoons, evidence of direct transmission between both species, and low amount of virus shed by skunks would likely impede the virus's ability to establish in wild populations. Similar results of an experimental trial with skunks were reported by Bosco-Lauth et al. (pre-print) (7). Of 6 striped skunks that were intranasally inoculated with approximately 10^5 PFU of SARS-CoV-2, three shed virus up to 7 dpi, the highest amount of which was 2.3 $\log_{10}$ pfu/swab. They also isolated virus from the nasal turbinates of 2/3 skunks euthanized on 3 dpi, similar to the vRNA detected in a nasal turbinate tissue sample of a skunk euthanized on 8 dpi in our study. Also similar to our study, all inoculated skunks seroconverted. Bosco-Lauth et al. (7) also inoculated three raccoons, none of which were positive by VI or RT-PCR. In our study, all of our inoculated raccoons seroconverted whereas none of the raccoons in Bosco-Lauth et al. (7) seroconverted. The reason for this difference is unknown but could be due to the use of different serological assays. Another important difference between these two studies is that we included naïve contact animals to test cage-mate transmission, which provided data on the potential for SARS-CoV-2 to circulate in striped skunk and raccoon populations.

We describe viral RNA using presence/absence data similar to methods for SARS-CoV-2 animal reporting by the USDA (41,58,59). We also provide data on the detection of infectious virus (by plaque assay) to assist in assessing the potential for transmission of SARS-CoV-2 in nature. In

some cases, virus quantification by PCR is used to estimate or determine virus quantities; however, this could lead to confusion as to how results translate to natural infections among wild and captive animals (55,60,61). In our study, virus was isolated from samples from two skunks (H1-L and H2-R) between 1-5 dpi; the corresponding Ct decreased notably between 3 and 4 dpi before steadily increasing (Supplementary Table 2). This trend may reflect diminished viral replication as Ct values decreased. While rRT-PCR is a sensitive diagnostic tool to evaluate for the presence of SARS-CoV-2, it should be used in conjunction with additional assays such as virus isolation to strengthen inferences about transmission potential and other epidemiological factors in wildlife and others.

As with many susceptibility trials with wildlife, especially those that require high containment housing, availability, logistical challenges, and animal welfare considerations often limit sample size (62,63). Given our studies small sample size and our animal sourcing, our findings may not readily translate into the susceptibility of wild populations due to factors such as senescence, immunocompetence (i.e., parasite burden, environmental conditions, gestation) and co-infections (e.g., canine distemper virus, parvovirus, etc.). Also, the rapid emergence of increasingly infectious SARS-CoV-2 variants in human populations presents new possibilities, such as increased transmissibility to previously marginally susceptible or unsusceptible species (64). Emerging variants of concern, B.1.1.7 and B.1.617.2, have been isolated from both companion animals (e.g., domestic dogs and cats) and captive wildlife (e.g., lions in a zoological institution), respectively (65–67). However, to date, no variants of concern have been isolated from free ranging wildlife, nor used in experimental infection trials of wildlife species, thus it is difficult to infer what impact these emerging variants will have on free-ranging raccoons and striped skunks.

Despite evidence of poor transmission, care should be taken to avoid transmission of SARS-CoV-2 to skunks and raccoons in a captive setting (e.g., zoological institutions, rehabilitation centers) where close encounter with individuals shedding different strains and high viral loads may influence outcomes. In this study, all raccoons and skunks seroconverted after direct inoculation with SARS-CoV-2, however only a small subset of these animals shed detectable viral RNA (n=7), and even less shed viable virus (n=2). Moreover, these results suggest that seroprevalence studies may be the most sensitive large-scale approach for determining COVID-19 exposure in susceptible wildlife contrary to current PCR-based animal surveillance in the US (59). However, the lack of viral shedding in raccoons or select skunks highlight that future wildlife surveillance studies should interpret antibody presence with caution, as seroconversion is not indicative of an animal having a profound role in the epidemiology of SARS-CoV-2.

While it seems unlikely for SARS-CoV-2 to circulate in raccoon and skunk populations, other taxonomically related species, such as several species of mustelids including various otters, weasels, badgers, and martens; especially species of particular conservation concern, like black-footed ferrets (*Mustela nigripes*), European mink (*Mustela lutreola*), giant otters (*Pteronura brasiliensis*), and sea otters (*Enhydra lutris*) have yet to be studied. Infection of highly susceptible species held in captive breeding programs, could result in outbreaks, hampering reintroduction efforts. For example, these concerns, in part, led to the majority of the captive breeding population of black-footed ferrets at the National Black-Footed Ferret Conservation Center outside Fort Collins, Colorado to be immunized with an experimental vaccine early in the COVID-19 pandemic (68). Continued global outbreaks of SARS-CoV-2 in farmed mink (69), sporadic reports of infection in domestic animals (36,70–72), and detected spillover into captive

and free-living wildlife populations (e.g., wild and escaped mink in Utah; (41,73), various species including tigers, gorilla, and otters in zoological collections; (44,74,75)) highlight that additional research including further exploration of the drivers, ecological pathways, and susceptibility of SARS-CoV-2 in wildlife, especially Musteloidea, are needed.

FIGURE LEGENDS AND TABLES

Figure 2.1 Conceptual model of the mechanisms of SARS-CoV-2 transmission from infected humans (direct transmission = solid orange arrows; and indirect transmission = dashed blue arrows) to susceptible wildlife (represented in greyscale). As depicted, SARS-CoV-2 shed by humans can be directly transmitted through activities that require handling and close contact (e.g., research and wildlife rehabilitation), or commercial operations (e.g., fur farms); however virus shed by humans could make its way into the environment via garbage (i.e., medical waste and household waste) and sewage. The solid gray arrow represents the establishment of SARS-CoV-2 in a wildlife species. The hypothesized spillback from this SARS-CoV-2 wildlife reservoir to susceptible human populations and other wildlife species is demonstrated by the solid green arrows.

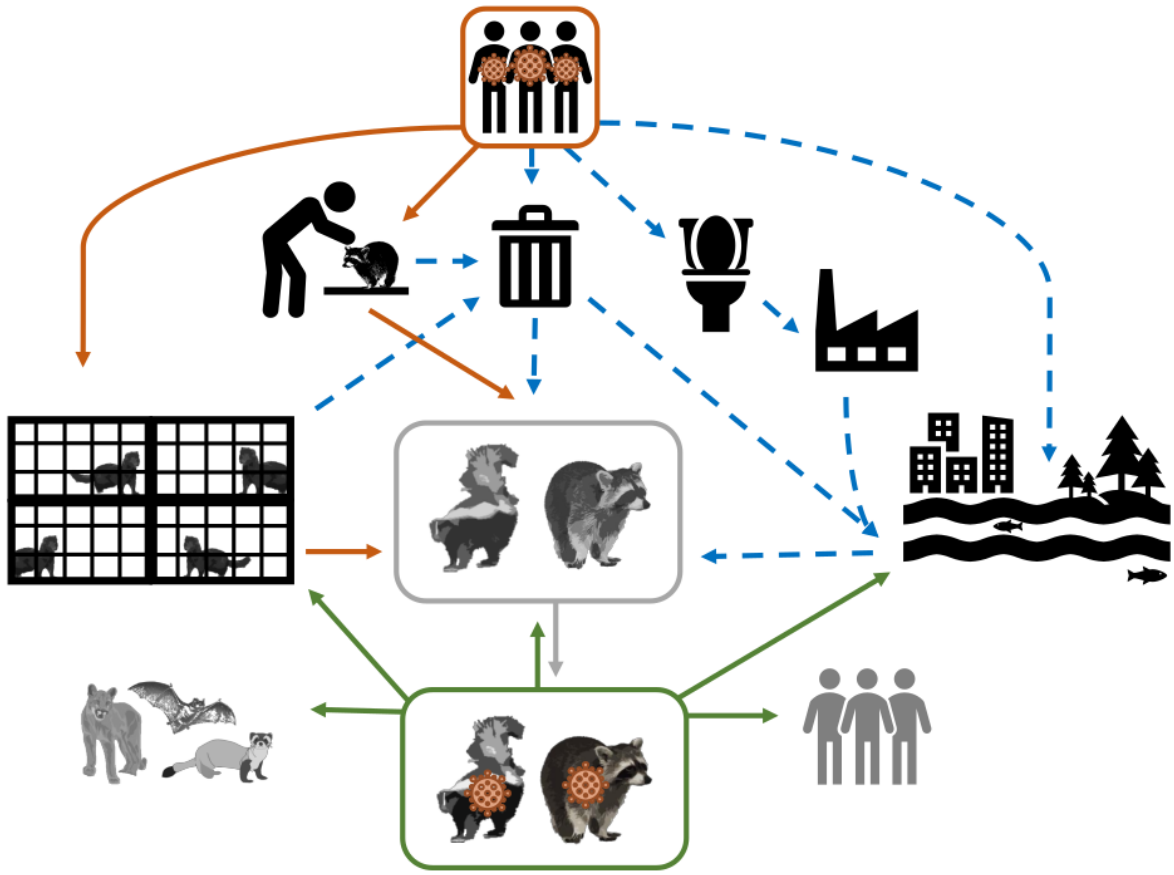
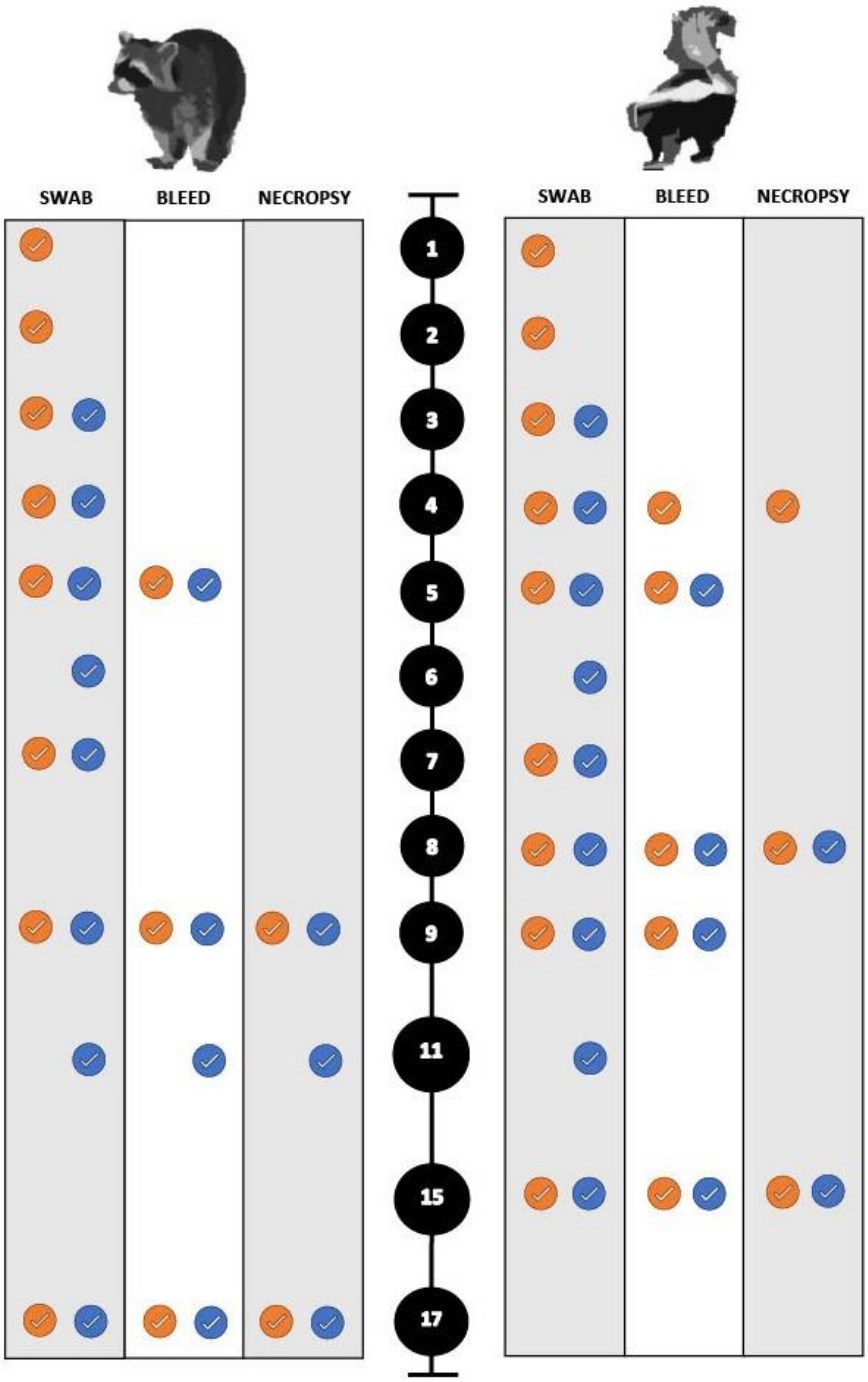


Figure 2.2 Sampling scheme and timing for the experimental SARS-CoV-2 infection trials of both raccoons and striped skunks. The black circles represent days post inoculation (dpi). The orange circles indicate directly inoculated (DI) animals. The blue circles indicate the direct contact (DC) animals.



Supplementary Figure 2.1 Agriculture Biosafety Level 3 (BSL-3Ag) room layout for both raccoon and skunk infection trials. The orange skunks represent the directly inoculated animals and the blue skunks represent the direct contact animals. The room's unidirectional airflow is represented by the arrow and did not recirculate.

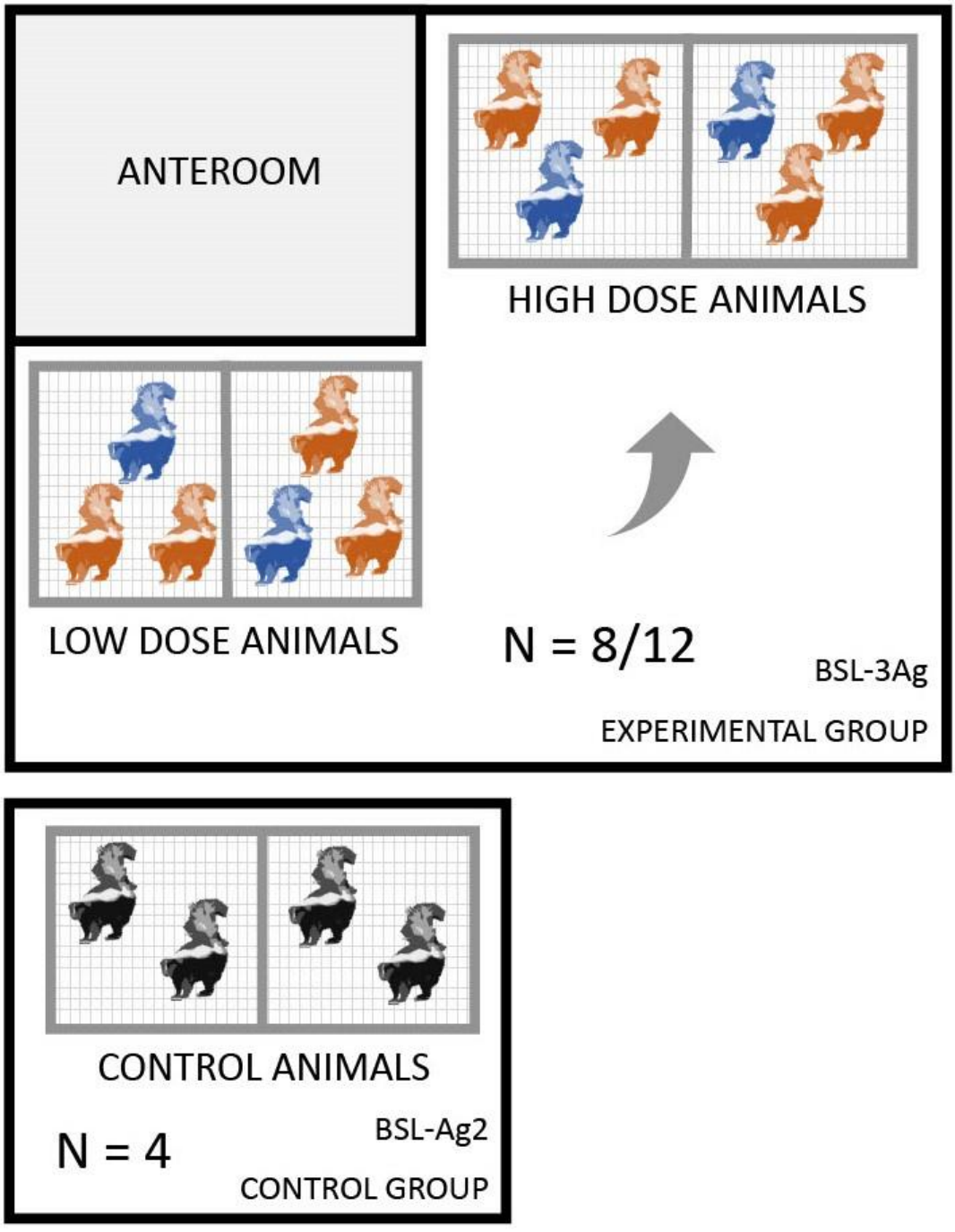


Table 2.1 SARS-CoV-2 virus isolation data for two striped skunks (*Mephitis mephitis*) that shed virus after intranasal inoculation. Values presented in plaque forming units (log_{10}/mL).

ID	Contact	Days Post Inoculation (dpi)				
Skunks*		**1**	**2**	**3**	**4**	**5**
H1-L	Directly Inoculated	2	3.2			
H2-R	Directly Inoculated			2.8	3.3	**0

*Both H1-L and H2-R were inoculated with a high dose (10^5 plaque forming units (PFU)) of SARS-CoV-2. No animals were found to shed virus after 5 dpi.

**The culture for H2-R developed cytopathic effect (CPE) on 5 dpi, however, no plaques were seen after staining.

Table 2.2 SARS-CoV-2 RNA detection in nasal swabs of intranasally direct inoculated (DI) and direct contact (DC) raccoons (*Procyon lotor*) and striped skunks (*Mephitis mephitis*) by real-time reverse transcriptase PCR (rRT-PCR).

DPI	**Raccoon***		**Skunk****	
Exposure	Direct	Contact	Direct	Contact
1	**3/8**		**2/8**	
2	0/8		**4/8**	
3	0/8	0/4	**3/8**	0/4
4	0/8	0/4	**2/8**	0/4
5	0/8	0/4	**2/6**	0/4
6		0/4		0/4
7	0/8	0/4	0/7	0/4
8			0/2	0/2
9	0/8	0/4	0/4	0/2
11		0/4		0/2
15			0/4	0/2
17	0/4	0/2		
Total Seroconversion	8/8	0/4	8/8	0/4

*Of the rRT-PCR positive raccoons, 2/3 belonged to the DI high dose groups and 1/3 belonged to the DI low dose group.

**Of the rRT-PCR positive skunks, 3/4 belonged to the DI high dose group and 1/4 belonged to the DI low dose group.

Supplementary Table 2.1 Serum neutralizing antibody development in striped skunks and raccoons intranasally inoculated with SARS-CoV-2, direct contact striped skunks and raccoons, and control striped skunks and raccoons.

ID	Contact	Detection*	DPI								
Skunks		**Method**	**Baseline**	**4**	**5**	**7**	**8**	**9**	**10**	**11**	**Study End**
C1-L	Control		1:4–1:8		1:4	**1:4**					
C1-R	Control		1:4					1:4–1:8			**1:4–1:8**
C2-L	Control		1:4–1:8					1:4			**1:4–1:8**
C2-R	Control		1:8	**1:4–1:8**							
L1-L	L-DI		1:4		1:4–1:8		**1:8–1:16**				
L1-R	L-DI		1:4–1:8	**1:4–1:8**							
L1-N	DC		1:4		1:4–1:8		1:4–1:8	**1:4–1:8**			
L2-L	L-DI		1:4		1:4–1:8			1:16–1:32			**1:32**
L2-R	L-DI	+; rRT-PCR	1:4					1:32–1:64			**1:64**
L2-N	DC		1:4					1:4–1:8			**1:4-1:8**
H1-L	H-DI	+; VI, rRT-PCR	1:4–1:8					1:32–1:64			**1:128**
H1-R	H-DI	+; rRT-PCR	1:4					1:32–1:64			**1:64-1:128**
H1-C	DC		1:16-1:32					1:32			**1:16-1:32**
H2-L	H-DI		1:4	**NS**							
H2-R	H-DI	+; VI, rRT-PCR	1:4–1:8		1:8		**1:16–1:32**				
H2-C	DC		1:4		1:4–1:8		**1:4–1:8**				
Raccoons		**Method**	**Baseline**	**4**	**5**	**7**	**8**	**9**	**10**	**11**	**Study End**
C1-L	Control		1:4						1:8		**1:4-1:8**
C1-R	Control		1:4						1:4-1:8		**1:4**
C2-L	Control		1:4						1:4-1:8	**1:4**	
C2-R	Control		1:4–1:8					**1:4**			
L1-L	L-DI		1:8					1:16			**1:32**
L1-R	L-DI		1:4					**1:8-1:16**			
L1-N	DC		1:4					1:4-1:8			**1:4-1:8**
L2-L	L-DI	+; rRT-PCR	1:4					1:8-1:16			**1:16**
L2-R	L-DI		1:8					**1:16**			

L2-N	DC		1:4–1:8	1:4	**1:4-1:8**	
H1-L	H-DI		1:4	1:8-1:16		**1:16-1:32**
H1-R	H-DI	+; rRT-PCR	1:4	**1:8-1:16**		
H1-C	DC		1:8-1:16	1:8		**1:8-1:16**
H2-L	H-DI	+; rRT-PCR	1:8	1:16-1:32		**1:64**
H2-R	H-DI		1:4	**1:16**		
H2-C	DC		1:8	1:4-1:8	**1:4-1:8**	

Table Footnotes: Bolded titers represent the terminal sample collected from an animal. End of study samples for remaining DI and DC raccoons were collected on DPI 17 and remaining control raccoons on DPI 18. End of study samples for remaining DI and DC skunks were collected on DPI 15 and remaining control skunks on DPI 14. *The "+" in the detection column indicates if SARS-CoV-2 was isolated in any animal via rRT-PCR or VI at any point of the study.

NS, No viable terminal sample was recovered due to hemolysis; DPI, day post inoculation; L-DI, Low Dose Directly Inoculated; H-DI, High Dose Directly Inoculated; DC, Direct Contact; VI, virus isolation; rRT-PCR, real-time reverse transcription PCR.

Supplementary Table 2.2 SARS-CoV-2 presence/absence Real-Time Reverse Transcriptase PCR results in striped skunks and raccoons intranasally inoculated with SARS-CoV-2 and direct contact striped skunks and raccoons.

ID	Contact	Swab	N1 Probe Ct Values									
Skunks			**1**	**2**	**3**	**4**	**5**	**6**	**7**	**9**	**11**	**15**
L1-L	L-DI	Nasal	NEG	NEG	NEG	NEG	NEG		NEG			
		Rectal	NEG	NEG	NEG	NEG			NEG			
L1-R	L-DI	Nasal	NEG	38.48		40.95						
		Rectal		37.84		NEG						
L1-N	DC	Nasal			39.44	NEG	NEG	NEG	38.62			
		Rectal			NEG	NEG	NEG	38.77				
L2-L	L-DI	Nasal	39.68	NEG	NEG	38.44	38.35		39.38	NEG		37.35
		Rectal	NEG	39.78	38.52	37.97	36.41		NEG	NEG		NEG
L2-R	L-DI	Nasal	38.46	31.41	NEG	NEG	33.95		37.95	NEG		37.92
		Rectal	38.93	NEG	39.66	NEG	NEG		NEG	NEG		NEG
L2-N	DC	Nasal			NEG	NEG	NEG	38.41		39.04	35.7	38.95
		Rectal			NEG	NEG	NEG	38.62	38.64	NEG	NEG	NEG
H1-L	H-DI	Nasal	35.29	31	29.27	33.87	29.87		36.22	NEG		NEG
		Rectal	35.46	NEG	NEG	39.74	NEG		NEG	35.92		38.63
H1-R	H-DI	Nasal	35.4	35.92	34.89	NEG	NEG		NEG	35.98		NEG
		Rectal	NEG	38.95	NEG	37.95	38.69		NEG	NEG		NEG
H1-C	DC	Nasal			37.21	NEG	NEG	39.47	NEG	NEG	39.04	37.55
		Rectal			35.39	NEG	38.63	NEG	NEG	NEG	39.56	NEG
H2-L	H-DI	Nasal	38.07	36.92	37.82	39.5						
		Rectal	NEG	36.82	37.63	NEG						
H2-R	H-DI	Nasal	35.39	34.19	29.86	26.81	30.55		37.36			
		Rectal	NEG	NEG	NEG	38.6	38.69		NEG			
H2-C	DC	Nasal			38.57	NEG	NEG	37.32	NEG			
		Rectal			39.64	NEG	NEG	NEG	NEG			
ID	**Contact**	**Swab**	**N2 Probe Ct Values**									
Skunks			**1**	**2**	**3**	**4**	**5**	**6**	**7**	**9**	**11**	**15**
L1-L	L-DI	Nasal	NEG	NEG	NEG	41.1	NEG		NEG			
		Rectal	NEG	NEG	NEG	NEG			NEG			

L1-R	L-DI	Nasal	39.47	NEG		NEG						
		Rectal		NEG		NEG						
L1-N	DC	Nasal			40.13	42.04	NEG	NEG	39.25			
		Rectal			40.71	NEG	40.68	42.01				
L2-L	L-DI	Nasal	42.02	NEG	NEG	39.99	36.8		NEG	40.56		41.01
		Rectal	40.26	NEG	NEG	NEG	39.05		NEG	NEG		NEG
L2-R	L-DI	Nasal	NEG	31.51	NEG	NEG	38.06		NEG	40.3		40.91
		Rectal	NEG	NEG	NEG	NEG	NEG		NEG	NEG		NEG
L2-N	DC	Nasal			40.83	NEG		NEG		39.14	39.7	44.86
		Rectal			NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
H1-L	H-DI	Nasal	35.15	30.5	28.68	33.89	31.4		36.97	41.99		41.61
		Rectal	37.76	NEG	42	40.68	NEG		NEG	41.33		40.91
H1-R	H-DI	Nasal	34.76	35.37	33.95	NEG			NEG	40.48		NEG
		Rectal	NEG	NEG	39.92	NEG	NEG		NEG	NEG		NEG
H1-C	DC	Nasal			39.74	38.91		NEG	39.19	NEG	39.97	NEG
		Rectal			37.13	NEG	39.89	NEG	NEG	NEG	NEG	NEG
H2-L	H-DI	Nasal	35.95	35.48	NEG	41.16						
		Rectal	NEG	39.58	NEG	40.26						
H2-R	H-DI	Nasal	38.16	33.78	32.01	27.12	32.32		38.82			
		Rectal	NEG	NEG	41.4	NEG	NEG		NEG			
H2-C	DC	Nasal			NEG	NEG	NEG	40.29	NEG			
		Rectal			NEG	NEG	NEG	NEG	41.13			

ID	**Contact**	**Swab**	**N1 Probe Ct Values**									
Raccoons			**1**	**2**	**3**	**4**	**5**	**6**	**7**	**9**	**11**	**17**
L1-L	L-DI	Nasal	35.41	35.69	36.16	36.29	37.86		36.19	37.95		36.31
		Rectal	34.32	35.78	NEG	NEG	37		35.99	36.79		NEG
L1-R	L-DI	Nasal	35.14	35.89	37.05	36.02	35.82		37.24	36.29		
		Rectal	NEG	NEG	NEG	38.99	NEG		NEG	36.19		
L1-N	DC	Nasal			38.65	35.96	35.59	37.22	36.37	35.45	37.84	35.36
		Rectal			37.13	NEG	38.02	NEG	39.04	NEG	38.71	38.81
L2-L	L-DI	Nasal	33.64	35.38	37.43	36.4	35.85		NEG	37.97		36.49
		Rectal	34.37	NEG	38.13	36.53	37.04		37.2	NEG		37.97
L2-R	L-DI	Nasal	35.2	35.01	NEG	38.62	NEG		34.14	37.2		
		Rectal	40.99	NEG	NEG	36.98	NEG		37.14	38.79		

L2-N	DC	Nasal			NEG	35.03	36.6	36.43	36.09	37.84	35.14	
		Rectal			NEG	37.04	36.32	35.75	36.16	NEG	37.64	
H1-L	H-DI	Nasal	37.56	36.98	34.31	35.9	35.28		NEG	36.2		35.31
		Rectal	34.13	34.7	NEG	NEG	36.95		35.88	34.42		37.25
H1-R	H-DI	Nasal	35.12	38.78	35.46	36.89	38.9		37.46	37.28		
		Rectal	34.71	NEG	35.47	37.15	37		38.91	NEG		
H1-C	DC	Nasal			36.37	35.87	38.47	36.96	38.96	38.76	38.07	36.41
		Rectal			NEG	NEG	NEG	38.13	38.25	NEG	35.77	36.16
H2-L	H-DI	Nasal	34.96	36.13	36.63	34.44	38.41		36	37.27		34.77
		Rectal	34.39	NEG	34.75	NEG	NEG		37.69	38.77		39.07
H2-R	H-DI	Nasal	34.82	36.46	NEG	35.64	37.29		34.85	NEG		
		Rectal	35.34	35.32	35.33	NEG	NEG		37.29	37.45		
H2-C	DC	Nasal			34.92	35.35	37.96	35.2	38.95	36.41	35.41	
		Rectal			35.47	38.1	NEG	36.81	36.81	38.77	38.98	
ID	**Contact**	**Swab**	**N2 Probe Ct Values**									
Raccoons			**1**	**2**	**3**	**4**	**5**	**6**	**7**	**9**	**11**	**17**
L1-L	L-DI	Nasal	37.36	39.31	40.28	38.54	40.81		38.51	39.54		NEG
		Rectal	NEG	41.1	NEG	38.14	NEG		NEG	40.24		NEG
L1-R	L-DI	Nasal	40.08	39.18	42.06	37.39	38.06		41.08	36.95		
		Rectal	41.5	NEG	40.62	39.8	41.68		39.21	37.46		
L1-N	DC	Nasal			40.3	38.87	36.35	38.76	38.19	40.28	38.11	39.65
		Rectal			NEG	NEG	NEG	41.99	40.97	42.78	NEG	NEG
L2-L	L-DI	Nasal	35.73	37.85	38.93	38.55	36.42		39.75	39.16		NEG
		Rectal	39.38	NEG	NEG	42.73	39.09		NEG	NEG		40.05
L2-R	L-DI	Nasal	37.58	37.86	40.88	42.03	36.76		36.78	40.63		
		Rectal	NEG	40.69	NEG	40.26	NEG		42.28	40.14		
L2-N	DC	Nasal			37.87	36.18	37.86	38.23	37.65	NEG	41.31	
		Rectal			NEG	NEG	NEG	NEG	NEG	42.04	NEG	
H1-L	H-DI	Nasal	36.51	40.47	37.48	36.62	36.55		38.95	38.57		NEG
		Rectal	41.99	NEG	38.22	NEG	38.53		NEG	37.4		NEG
H1-R	H-DI	Nasal	35.61	40.67	41.08	37.07	39.47		38.99	36.92		
		Rectal	NEG	42.79	NEG	NEG	42.05		37.3	38.99		
H1-C	DC	Nasal			NEG	41.4	40	37.08	41.66	39.73	NEG	39.13
		Rectal			NEG	40.33	NEG	NEG	39.11	NEG	37.26	40.12

H2-L	H-DI	Nasal	35.48	38.53	40.53	36.44	37.1		37.76	NEG		NEG
		Rectal	40.01	40.25	39.24	38.11	40.68		40.2	43.7		42.02
H2-R	H-DI	Nasal	38.66	38.75	41.87	37.39	37.58		37.85	NEG		
		Rectal	NEG	38.63	40.82	40.67	NEG		NEG	NEG		
H2-C	DC	Nasal			36.6	36.63	39.2	36.6	40.93	NEG	39.88	
		Rectal			39.28	NEG	36.98	38.22	38.22	NEG	41.07	

Table Footnotes: The N1 and N2 primer/probe had to have a cycle threshold (Ct) of ≤35 to be considered positive (**GREEN**) for the presence of SARS-CoV-2 RNA. Samples evaluated that resulted in a Ct of >35 for both probes were considered negative (**RED**) and samples with a Ct of ≤35 for one probe and a Ct of >35 for the other probe (**YELLOW**) are also considered negative.

NEG, Negative; DPI, day post inoculation; L-DI, Low Dose Directly Inoculated; H-DI, High Dose Directly Inoculated; DC, Direct Contact; rRT-PCR, real-time reverse transcription PCR.

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CHAPTER 3

HUSBANDRY AND TECHNIQUES FOR MANAGING NORTHERN RACCOONS (*PROCYON LOTOR*) AND STRIPED SKUNKS (*MEPHITIS MEPHITIS*) IN A HIGH CONTAINMENT FACILITY

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ABSTRACT

Striped skunks (*Mephitis mephitis*) and raccoons (*Procyon lotor*) are notorious for serving as reservoirs of pathogens that circulate among other wildlife, domestic animals, and humans. Both skunks and raccoons are opportunistic, omnivorous generalists that are native to and widespread in North America. Despite their prolific nature and relevance to zoonotic diseases, their status as terrestrial rabies vectors and reputation for aggressive behavior have precluded them from being explored as viable research species. The husbandry of animals in a research laboratory often is vastly different than in a zoologic or rehabilitation setting due to facility construction, animal welfare regulations, biosafety restrictions, and the investigation goals. Herein, we describe the husbandry and handling techniques developed and effectively utilized for both striped skunks and raccoons in Biosafety Level 3Ag conditions. We present an experimental infection trial of SARS-CoV-2 susceptibility in striped skunks and raccoons at the University of Georgia's Animal Health Research Center as a case study on how to implement these techniques.

INTRODUCTION

Wildlife can be extremely complex to study *in situ* due to life history traits of a species (e.g., home range, habitat selection, social groups), accessibility, and the complexity of their natural environments. A controlled captive setting can eliminate variability, offer the ability to control for extraneous variables, and facilitate animal handling. For example the pathogen dynamics of wildlife species, such as intra- and interspecies transmission, factors facilitation transmission, and duration and quantification of shedding are often best investigated in a laboratory setting.[41] Research facilities with the resources to appropriately contain these species allow researchers to objectively explore questions otherwise impossible to address in a natural setting (i.e., determine

pathogen and toxin susceptibility, explore effect of various nutritional supplementation, allow for the evaluation of novel vaccinations, etc.).[45] Well justified and ethical wildlife research within these facilities can allow for the safe exploration of these topics, while inflicting minimal to no impact on wild populations. Often times the use of wildlife instead of traditional laboratory animal species (e.g., purpose bred beagles, mice, etc.) in research is more appropriate, however few resources are available for the maintenance of most wildlife species in a research setting.

Wildlife are an important indicator of human, animal, and environmental health. Practices linked to globalization and industrialization often promote human interactions with wildlife (e.g., wildlife trade, consumption of bushmeat, deforestation, agriculture), creating opportunities for novel pathogens (e.g., Nipah virus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV 2), Ebola virus, etc.) to emerge in wildlife, domestic animal, and human populations in addition to facilitate on-going transmission of endemic pathogens (e.g., *Salmonella spp., Leptospirosis spp., West Nile virus, Francisella tularensis, Yersinia pestis*, Hanta virus).[32] In addition to facilitating the spread of pathogens, global development has heavily impacted once natural environments with toxins and contaminates. Free-roaming wildlife species can be utilized as indictors for anthropogenic impacts that have resulted from agriculture, industrialization, and general human development. For example, the deleterious estrogenic effects of the pesticide 1,1,1-trichloro-2,2-bis(p-chlorophenyl) ethane (DDT) were first highlighted due to reproductive abnormalities documented in the American alligator (*Alligator mississippiensis*).[18,65] More recently, marine wildlife populations are being explored as sentinel species for impacts of microplastics and associated toxicities.[11] Key One Health issues, such as antimicrobial resistance (AMR), also utilize wildlife populations from a variety of taxa to study the distribution, impact,

and spread of antimicrobial resistance, to not only better understand AMR but also quantify anthropogenic impacts on the environment.[20,56] Despite consensus that wildlife research is instrumental to a One Health approach, which can be defined as an approach to ensure the well-being of people, animals and the environment through collaborative problem solving, information is lacking on husbandry and handling practices of wildlife within research facilities.[40]

Two wildlife species of importance in North America are the raccoon (*Procyon lotor*), also referred to as the northern raccoon or common raccoon, and the striped skunk (*Mephitis mephitis*). Procyonids and mephitids are important hosts for pathogens of significance, both to human health and to wildlife populations. Striped skunks and raccoons are abundant and ubiquitous throughout much of North America. [25,69] Both species are furbearers, thus are often trapped or hunted by sportsman. Raccoons and skunks also commonly present to wildlife rehabilitation centers for treatment and are then reintroduced into natural environments.[31,38] In addition, several US states allow for the legal sale of both of these species as pets.[37] Most importantly, these two species thrive in urban and suburban landscapes, are now habituated to food and shelter near human homes, and often come into direct or indirect contact with humans, their waste, and domestic animals; as such, may people perceive these species as peridomestic pests. These frequent interactions allow both skunks and raccoons to act as a bridge between the environment, humans, companion or production animals, and other wildlife species.

Due to the pervasive nature of raccoons and skunks, they are excellent indicators of human, animal, and environmental health.[12] Both species have been implicated as competent sentinels

for environmental contamination due to both trophic position and utilization of their environment.[15,24,28,47] They are also susceptible to various pathogens while playing a potential role in disease transmission to conspecifics and other wildlife species (e.g., canine distemper virus, rabies virus, feline and canine variants of carnivore protoparvovirus) across North America.[2,3,16,17,29,66] As reservoirs of pathogens that have substantial impacts on other wildlife species, domestic animals, and humans (e.g., *Baylisascaris procyonis, Bartonella spp., Leptospirosis ssp.,* Aleutian disease virus) and incidental hosts for several zoonotic pathogens (e.g., *Salmonella ssp., Toxoplasma gondii*), skunks and raccoons are valuable species to investigate pathogen dynamics within a laboratory setting.[6,14,30,35,43,62] Currently, no literature is available that is dedicated to the housing, husbandry, and handling of either species in a biosecure research setting.

TAXONOMY AND NATURAL HISTORY

Musteloidea is one of the most abundant superfamilies within the order Carnivora. This clade comprises 30% of the species diversity found within Carnivora with 85 known living species in 33 genera and over 400 described extinct species.[36,59] The Musteloidea superfamily is composed of the families Mustelidea (e.g., otters, martens, badgers), Ailuridae (e.g., red pandas), Mephitidae (e.g., skunks and stink badgers), and Procyonidae (e.g., raccoons, coatimundis), all of which inhabit a wide variety of ecological niches.[36] While the exact time period is still debated, phylogenetic studies have demonstrated that these four families originated from the same ancestor.[58] This shared ancestor has likely lead to similar disease susceptibility within various musteloidea families.[1,60,61]

Striped Skunks

Striped skunks (*Mephitis mephitis*) share the family Mephitidae with 11 other species of skunks.[7] Striped skunks are the approximate size of a house cat and are notorious for their fear response, which involves ejecting a foul-smelling substance from a paired anal glad.[67] Skunks have small ears, stocky legs with 5 digits on each foot, and a dense coat, similar to many Musteloidea species. They have long front claws that are used for digging and foraging, in addition to well-developed canines (dental formula: I 3/3 C1/1 P3/3 M1/2 = 34).[21,44] Striped skunks are crepuscular to nocturnal and, as generalist omnivores, occupy a wide habitat range. Skunks generally are considered solitary; however, studies have shown that adults and juveniles can cohabitate dens. This is a predominantly female trait in which usually no more than two individuals are found.[53] Striped skunks are known to have relatively small home ranges (i.e., 8 to 300 ha) that vary in size depending on season and resources.[13,54] This species has been anecdotally reported to make enjoyable companion animals when appropriately socialized, however they are believed to become more fractious with age. [21,22,31]

Northern Raccoons

Northern raccoons (*Procyon lotor*) are one of the 14 living species within the family Procyonidae.[59,60] Similarly to striped skunks, they have an extensive natural range that extends throughout most of North America and into Central America; there are also feral colonies in parts of Europe and Asia.[69] Raccoons are medium-sized, stout mesomammals that have a distinctive black mask across their eyes, erect triangular ears, thick coats composed of gray to brown fur, and a striped tail (dental formula: I 3/3, C 1/1, P 4/4, M 2/2 = 40).[34,63] Raccoons, like striped skunks, can successfully inhabit various landscapes, including urban settings.[26] They are

notorious for their dexterity and intelligence, which is often used to scavenge anthropogenic waste. Raccoon home ranges are not vast (e.g., 5 to 300 ha), with population densities varying depending on seasonality, landscape composition (i.e., urban vs natural), geographic location, and resources availability.[49,50,55] Raccoon densities increase significantly with the availability of food sources in urban/suburban (e.g., trash cans and feral cat feeding stations) and agricultural areas (e.g., crop fields).[8,49,50] They are often perceived as solitary animals, but most have interconnected and expansive social networks, particularly during the winter and mating seasons.[29,49,52]

CONSIDERATIONS FOR WILDLIFE IN RESEARCH FACILITIES

Biosecurity practices (i.e., engaging in minimal direct contact, wearing gloves, avoiding cross contamination of equipment and caging) often are standard considerations when working with both captive and free-roaming wildlife; however, more stringent rules and regulations may apply depending on the intended use of the animal and available facilities. The subject of the research (e.g., infectious agents, parasites, toxins, etc.) often dictates the level of biosecurity necessary via risk assessment. A biological risk assessment encompasses 1) identification of hazards and risks, 2) evaluation the risks, 3) development of a risk control strategy, 4) the selection and implementation risk control measures, and 5) reviewing the risks and risk control measures.[68] The results of this evaluation dictate the required Biosafety Level for the study, which will affect both approvals needed, laboratory and animal housing facilities, personal protective equipment of study participants, and animal handling protocols.

While some wildlife research may be acceptable to perform in outdoor facilities (e.g., nutritional, immobilization, and behavior studies), pathogen or toxin research often requires animals to be held in containment facilities. Moreover, wildlife housed in containment facilities often have different husbandry and handling needs then animals held in captivity at zoological institutions and wildlife rehabilitation centers. For example, raccoons often are hand-raised and group housed in rehabilitation centers and once weaned, are moved as a group to outdoor facilities with minimal contact with humans to discourage habituation. In contrast, a research animal is housed either solo or in small groups as dictated by it's natural history and may handled multiple times in a week depending on study design. Both in zoos and rehabilitation centers, raccoons and skunks are afforded outdoor spaces where providing environmental enrichment is relatively simple. However, in containment all lighting, housing, and substrates are artificial, to allow for complete decontamination both before and after introduction of the animal. Minute details like room color and personnel uniforms (i.e., PPE requirements) are highly regulated. Animal care, husbandry, nutrition, and enrichment must be balanced against the strict guidelines and personnel limits of the research facility. In addition, animal care staff often require additional training on the behavior, biology, handling, and husbandry of wildlife species.[46]

The critical ecological role of raccoons and skunks in North American ecosystems and their role at the human-domestic animal-wildlife interface justify their use in captive studies to explore topics such as pathogen dynamics and toxicant effects. Herein we describe best practices for housing and handling raccoons and striped skunks within both ABSL-2 and BSL-3Ag containment facilities for research purposes. Our methods were tailored to a susceptibility trial study for SARS-CoV-2 but are broadly applicable to research involving raccoons and skunks

within a containment facility.[23] In the aforementioned study, control animals were held under almost identical husbandry conditions (i.e., the same diet, housing, grouping, enrichment, and handling practices) as a ABSL-2 level to reduce variability within the study. With regards to animal handling, the most notable distinctions in the BSL-3Ag room were the required PPE for researchers and animal care personnel (e.g., additional layers that limited mobility and a Powered Air Purifying Respirator). This manuscript provides guidance and highlights the unique challenges of managing these wildlife species in a biosecure research facility. All procedures were reviewed and approved by the University of Georgia IACUC committee protocol # (A2020 04-016) and the Institutional Biosafety Committee (protocol # 2020 0048).

HUSBANDRY AND HANDLING

The Study

Sixteen raccoons and sixteen descented skunks ranging from 9 to10 weeks old were acquired from captive breeding facilities at separate intervals. Upon arrival, the animals were allowed to acclimate for two weeks in a BSL-2 facility prior to the transfer of experimental animals (n=12 for each species) into the BSL-3 facility; control animals (n=4 for each species) remained in the BSL-2 with similar husbandry as experimental animals. Once in the BSL-3 facility, experimental animals were intranasally inoculated with SARS-CoV-2, the causative agent of COVID-19, to evaluate species susceptibility to infection. The trial was performed first on raccoons, then the same room and equipment were decontaminated and used to house the skunks. In total, raccoons were housed in the BSL-3 facility for 17 days and skunks were housed for 15 days.[23]

Biosafety Facilities

The Veterinary BioResources Facility (VBF), Athens, GA USA was utilized as a ABSL-2 facility to house study animals upon arrival and the subsequent two-week acclimation period. Only the control skunks (n=4) remained at VBF for the entirety of the study; however, the husbandry, including enclosures and handling, were similar to BSL-3Ag housing and procedures. The remainder of the animals (n=16 raccoons and n=12 skunks) were transferred to an BSL-3Ag facility at the beginning of the study where they remained until the study conclusion. The BSL-3 Ag facility utilized for this study was the Animal Health Research Center (AHRC), Athens, GA USA.

Housing Structure and Maintenance

Ideally, wildlife enclosures mimic a species' natural habitat while providing areas that meet specific physical, social, behavioral, and psychological needs. The Association of Zoos and Aquariums (AZA) Taxon Advisory Group (TAG) for both Procyonidae and Mustelidae advise that enclosure size should be determined by three factors: species average head and body length, typical home range size and activity level, and professional experience with the species.[4,5] Optimal enclosures for musteloidea species are often comprised of a large outdoor exhibit with copious areas to den, explore, and, in the case of raccoons, climb; with an additional indoor enclosure serving as night quarters/holding area. AZA TAG has several equations to calculate spatial requirements for each species. For long term housing purposes, AZA TAG recommends a minimum floor space of between 29 m^2 for a single striped skunks and 43.6 m^2 for a pair of raccoons, in addition to at least 3 m of vertical space.[4,5]

Enclosure complexity is also paramount to meet a species physical, social, behavioral, and psychological needs. Raccoons, more so than skunks, require vertical space to engage in species-appropriate behavior (e.g., climbing, denning, foraging, socializing). Nesting areas/boxes for raccoons should consist of both elevated and terrestrial options with at least one location of sufficient size to accommodate all animals in a given group to den together.[5] Skunks, like raccoons, should have at least one nesting area large enough to fit all animals in the enclosure if they choose to sleep together; however all nesting areas/boxes should be terrestrial and at least large enough to allow the animal to turn around.[4] Both species forage mostly on the ground, thus feeding and water stations should be easily accessed from the floor.

Due to the nature of containment facilities and biosafety requirements, research animals generally are housed solely indoors. Indoor housing recommendations for skunks and raccoons dictated by the minimum standards for wildlife rehabilitation are 0.9x0.9x0.9 m to 0.6x0.9x0.9 m, respectively.[42,60] Basic indoor housing recommendations for musteloidea recommend a structure that is completely enclosed with thick gauge fencing that cannot be destroyed by chewing as well as a denning area, a floor that can be easily disinfected on a regular basis, ample space to place food and water bowls on the ground, and various enrichment items.[1,60] Most AZA TAG recommendations for enclosure substrate (e.g., shavings, pebbles) and enrichment items (e.g., logs, branches, rocks) consist of organic materials that would be inappropriate for a research setting which often require surfaces to be easily cleaned and disinfected regularly.[4,5] When housing dexterous species, such as raccoons, extra precautions must be taken to ensure lock latches are secured and no large gaps are present in the enclosure. Additional considerations in a research setting include adding or modifying aspects of the enclosure to ensure safe regular

handling of research animals (i.e., specially designed enclosures that funnel animals to small cages that can be sealed off) and adapting practices to fit the study-specific objectives.

Our research took place within two separate vivariums (e.g., both within in BSL-3Ag) that accommodated large enclosures. The area of the first vivarium used for control animals was approximately 22 m^2 and the area of the second vivarium used for experimental animals was 76 m^2; both had ceilings that were 3.7 m in height. Each vivarium contained either one (control room) or two (experimental room) modified modular kennels manufactured by Britz & Company (model number 22000 identification number 2734 [BH, Inc., WY, USA]). When indicated and if available, squeeze chutes may be incorporated into the housing; however, this feature was not available for our study. The dimension of the enclosure was approximately 3x1.5x21m and each enclosure had 2 distinct kennels that were approximately 1.5x1.5x2.1 m. If needed, kennels can be connected via a door in the partition. Figure 1 demonstrates the partition in the open position; however, in our study, we elected to keep this partition closed due to study design.

Prior to modification, the kennels were stainless-steel with walls constructed with 3.81 cm (1.5 in) x 16-gauge steel wire mesh and 2.54 cm round stainless-steel tubing, sliding door panels, but lacked ceilings. For our work, kennels were fully enclosed with ~5x5 cm (2x2 in) 14-gauge wire 60.96x182.88 cm (24x72 in) fence panels that were sistered and secured to the top of the kennel with ~5 cm outdoor nylon cable ties (Utilitech™, LG Sourcing, NC, USA). To reinforce each kennel, 0.9 m (3 ft) poultry fencing (Tenax®, MD, USA) was firmly attached to the inside of the enclosure ceiling and stationary walls with ~5 cm outdoor cable ties (Utilitech™). Each panel of poultry fencing was overlapped ~10 cm and secured to another panel with ~5 cm cable wire. The

poultry fencing not only created an additional barrier against potential escape, but it also provided an easy substrate for raccoons to climb, complementing their enrichment.

A large canvas hay bag (Derby Originals, OH, USA) was hung by a chain and carabiner and used as a hammock that provided a denning area in each raccoon enclosure. In addition, one small animal carrier (24" Ruffmaxx, Petmate, TX, USA) was placed in each raccoon enclosure to provide additional denning options. Housing conditions for skunks were almost identical to raccoons; however, no hammocks were provided as skunks are not proficient climbers and are known to prefer ground level dens.[31] For skunks, small animal carriers were utilized as sole denning areas and one carrier per animal was provided within the enclosure. However, it was not unusual for skunks to share a den, as observed when researchers were present. Both species were offered a variety of objects for enrichment, including Kong Classic Dog Toy (Kong Company, CO, USA), Flexi-Keys (Bio-Serv®, NJ, USA), which were free in the cage, and a cotton horse lead and a metal tringle with washers on a stainless-steel chain that were both hung in each enclosure. Different toys were removed and reintroduce sporadically to keep the animals active and engaged.

Each kennel had textured slatted fiberglass flooring (BH, Inc), which allowed for easy waste removal and disinfection. Once a day, each kennel floor was rinsed with high-pressure hoses, allowing the waste to wash through the slats on the floor and into the vivarium drains. Cleaning could be performed with the animals in the enclosures, since raccoons and skunks moved away from the doors and remained distanced from humans. During cleaning, raccoons generally climbed up the side of the cage to avoid the spray of the hose. Skunks usually hid in their

carriers, which were latched and positioned away from the hose within the enclosure. Occasionally, skunks used one of the small carriers as a latrine, thus carriers also were cleaned daily. Although raccoons in the wild form latrines, they did not develop a regular latrine during the study. Each kennel used in this project housed up to four juvenile animals of each species comfortably and without incident.

Environmental Parameters

Animals were maintained at ~21˚C and 50% humidity with a 12:12 hour light-dark photocycle within the BSL-3Ag facility. The control skunks at the ABSL-2 facility were kept at ~18.7˚C with a 12:12 light-dark photocycle; however, the humidity was variable with an average of ~74% (range of 72.98% to 77.95%) .

Group Housing and Animal Behavior

Both raccoons and skunks have complex social systems, which means they should be kept in carefully monitored groups when held in captivity.[26,60,69] Because adult skunks and raccoons can be difficult to integrate to a new group, acquiring young animals is preferred. The use of young animals can reduce intraspecies aggression, making this age preferred for short studies in containment.[22,42] However, once sexually mature, males of both species are solitary and can engage in aggressive behavior. Even as juveniles, male skunks can be aggressive to conspecifics.[22,44] Our study used juveniles that never reached sexual maturity; raccoons were housed in same sex groups and skunks in mixed sex groups and there was minimal intraspecies conflict. However, in general, male skunks were the sex and species group that exhibited the most aggression towards cage-mates.

Due to the short duration of our study, there was inadequate time to condition animals for voluntary sample collection. Furthermore, PPE protocols necessitated by use of an infectious, zoonotic agent in our study (e.g., full-body Tyvek suits, Tyvek aprons, boots, and Powered Air Purifying Respirators (PAPRs)), limited researchers' mobility, the ability to communicate, and dexterity. Required PPE can also be perceived as frightening to the animal, thereby increasing its stress levels. However, even in this challenging setting, researchers and study animals can still benefit from the use of positive reinforcement to increase acclimation/reduce stress as detailed below.

Nutrition and Enrichment

As generalist omnivores, both skunks and raccoons should be fed a commercially available dog or specialty omnivore diet in captivity. During this study, both species were fed once a day with a commercial omnivore diet (Mazuri® Omnivore Diet, Purina Mills, LLC., MO, USA), water *ad libitum*, and provided with supplemental food items (i.e., fruits, vegetables, and eggs) twice daily for enrichment to encourage natural forging behaviors.[22,33,51,60] Food was placed in two approximately 2 L stainless steel bowls (Vollrath®, WI, USA) per enclosure. All animals were provided marshmallows, directly handed to individual animals via tongs during cleaning and handling to positively reinforce the presence of researchers and sporadically throughout the day to encourage foraging. Water was provided in two approximately 4 L no-tip rubber bowls (Fortex®, PR, USA) both for drinking and for raccoons to display food washing behavior which was regularly observed.

Identification

Raccoons and skunks are often individually identified by microchips and/or external markers (e.g., ear tags, collars, ear notches) in a captive setting. For our study, we chose a method that would allow us to identify individuals from a distance without handling and did not require an additional anesthetic period or recovery. Animals were housed in groups of up to four individuals that were identified by the area clipped of hair at their first anesthetic event; the right rump, left rump, or midline on the caudal dorsum. An animal's unique identifier was based on the anatomic region clipped (e.g., left rump, center rump, right rump, no clipped area), treatment group, and kennel position (i.e., skunk high-dose kennel 1 right-clipped [SH1-R]). Each time an animal was handled for invasive sampling where full immobilization was required (i.e., approximately every ~4 to 5 days for ~2 weeks), the patch was reclipped to ensure hair growth did not obscure its location. This method allowed for an easy, low-stress, inexpensive, and painless way to identify each individual; however, this method is less indicated in studies that do not regularly handle the animals.

Handing and Restraint

Skunks - Techniques recommended to physically restrain skunks are similar to those used with domestic ferrets.[22,44] The skunk should be "scruffed", an action that requires all the extra skin on the cranial dorsum to be secured firmly in one hand, while the second hand is used to lightly restrain the base of the tail to prevent the animal from adducting. While holding the animal, the head should be adequately immobilized by ensuring that all loose skin around the head and shoulders is held within the hand scruffing the animal. When trying to first grab an animal from the ground or from a carrier, it is easiest to lightly restrain their tail and slowly place the other

hand on their dorsum to scruff them. Skunks will not hesitate to bite a researcher who is restraining them inappropriately. Regardless, the handler should wear appropriate PPE (exam gloves under bite gloves) when handling animals. The bite gloves we used were made of HexArmor's exclusively-licensed SuperFabric® which is rated at American National Safety Institute/International Safety Equipment Association (ANSI/ISEA) cut level A9 resistance; highest cut protection available in the industry to protect the entire hand. The model glove used was Hercules® 400R6E (HexArmor®, MI, USA). Individually fitting gloves to hand size of researchers that will be handling animals is recommend because improperly sized gloves further limit dexterity and can lead to avoidable injuries. At times, heavy duty leather gardening gloves were used to handle skunks. Some juvenile skunks were easier to restrain, especially after given a sedative, thus bite gloves were not always used. In addition, restraining the animal against another surface can prevent it from scratching and tearing the PPE and may make it feel more secure.

Raccoons – Raccoons tend to climb up the walls of their enclosure to increase distance from handlers. Handlers can use this to their advantage and lightly pin the animal to the wall of the enclosure with one hand at the base of the neck to restrict the movement of their head, and one hand on their rump as to prevent them from backing out of the hold. At this point the animal is restrained appropriately for the administration of injections or oral liquids medication. Further manipulation, including moving them up away from the wall, will require sedatives or anesthetics. When sedated, raccoons can be gently removed from the wall while the handler maintains control of their head (i.e., the hand responsible for restraining the rump, is moved to the front of the animal to help control the head) and reposition it as needed. Care should be taken

to unhook animals' nails from wall structures to avoid injury to the animal. Raccoons will bite when threated, thus control of their head must be maintained at all times.

To facilitate swab sampling, a ~60x60 cm piece of Plexiglas with a 3.8 cm (1.5 in) hole was placed at chest height on the inside of the front wall of the kennel. Researchers would then place the animal's nose into the small Plexiglas hole and a nasal swab sample was taken. The Plexiglas prevents the animal from grasping any of the cage bars, netting, or swab while also preventing the animal from biting the swab or fellow handler.

Chemical restraint

Numerous immobilization protocols are reported for raccoons and skunks in the field; however, most of these involve the use of ketamine, a non-reversible and long acting anesthetic drug.[9,10,19,48] We elected to utilize 0.05 mg/kg dexmedetomidine and 0.2 mg/kg of butorphanol intramuscularly (IM), which led to fast acting, reversible anesthesia of short duration and was adequate for the minimally invasive procedures we performed (e.g., venipuncture, shaving fur; Francisco and Hernandez, unpublished data). Dexmedetomidine and butorphanol maintained a desirable plane of anesthesia (i.e., unresponsive to stimuli, relaxed muscle tone, and lack of righting reflex) for approximately 15 to 25 minutes. For longer or more stimulating procedures, supplementation with other anesthetics (e.g., ketamine) or additional light restraint should be considered. Personal protective equipment constraints prevented the use of a stethoscope and decontamination procedures in BSL-3Ag facilities precluded the use of other standard monitoring equipment (e.g., pulse oximeters). Thus, anesthetized animals were monitored by assessment of pulse rate and quality and respiration by placing a palm lightly around the upper

thorax, observing the thorax, and palpating peripheral pulses. Once animals were sampled, reversal agents were administered. Animals were allowed to recover in their enclosure on top of a dry, clean, disposable surface (e.g., absorbent single-use pad). The recovery was visually monitored until the animals were ambulatory.

Not all handling protocols require the animal to be completely immobilized. For a minor procedure such as rectal or nasal swabbing, sedatives can be considered. We utilized oral trazodone, an anxiolytic which causes mild sedation. It is not palatable, and the methods commonly used when dosing domestic animals (i.e., pills hidden inside palatable food items) were unsuccessful in our study. Due to the dexterity of both species, they easily removed the unpalatable items from the treats (e.g., pill pockets of various flavors, squeeze cheese, and marshmallows). The most efficient technique for drug delivery was to crush trazodone tablets and mix them in a liquid vehicle, allowing them to dissolve in water. Alternately, flavored trazodone can be formulated at a compounding pharmacy. The dose that we found useful for light sedation and anxiolysis for both raccoons and skunks was 45 mg/kg (Francisco and Hernandez unpublished data). It took approximately 30 to 40 minutes after administration for animals to be sedated enough for non-invasive sampling (i.e., light restraint that allowed for nasal and rectal swabbing). Raccoons remained sedated between 1 to 4 hours but were able to eat and ambulate well. Skunks were more profoundly affected by trazodone and remained sedated for 4 to 6 hours after dosing. The length of anxiolysis and sedation seen in both species rendered by trazodone administration made the drug a useful tool to facilitate light restraint for painless procedures or transportation.

DISCUSSION

The techniques and husbandry described herein allowed for the safe handling of raccoons and skunks in biosecure facilities during an approximately four-week study on SARS-CoV-2 susceptibility. The housing recommended here is directed towards animals to be managed for short periods of time. Long term projects that span many months to years should consider enclosures with more floorspace, similar to AZA outdoor recommendations, and behavior changes that accompany sexual maturity.[4,5,42] All husbandry and handling practices used for animals in BSL-3Ag, were also successfully implemented for non-infected control animals housed outside of high containment (n=4; for both species); thus, practices recommended herein are appropriate for both BSL-2 and BSL-3 research.

The modified kennels securely contained the animals. One raccoon escaped its enclosure the first afternoon the animals were transferred to the BSL-3Ag facility by slipping through a small (~10cm) gap in the retrofitted ceiling after squeezing through the poultry fencing. The animal was recaptured by placing food into a small animal carrier as bait; the carrier was closed when the animal entered, and the entire carrier was placed into the enclosure for release. Further escapes were prevented by placing cable ties in more regular intervals (~5cm) at the seams of the poultry fencing throughout the entirety of all enclosures. Group housing of both species allowed for appropriate species interactions (e.g., communal denning, explorative behavior), with only a single instance of intraspecies aggression noted during the acclimatization phase of our study—a male skunk was aggressive towards a female resulting in superficial skin laceration of her rostrum. The male skunk subsequently was assigned to be a control animal and thus was singly housed in VBF, the ABSL-2 facility. Single housing of social animals requires justification and

approval by the IACUC and aggression is an adequate exemption. Strategically shaving the fur on subjects allowed for the prompt and reliable identification of group housed animals. Lastly, the techniques recommended for restraining raccoons and skunks allowed for rapid sampling or immobilization with minimal stress to the animals and considerable human safety.

As the wildlife-domestic animal-human interface continues to expand, the need to assess and establish wildlife species as animal models also may increase, with peridomestic species being logical choices for experimental manipulations. However, the use of wild-caught animals in research presents a variety of ethical dilemmas and logistical challenges. Free-living wildlife often have an unknown medical history and health status and their displacement from the environment may disrupt social structures. Further, in some cases, target species may be of conservation concern and thus should not be removed from the wild. To circumvent these concerns and ensure minimal impact on wild populations, the use of captive-bred wildlife is encouraged.[46] Our use of captive-bred animals likely facilitated handling as they were at least minimally habituated to humans; however, these animals were by no means considered tame thus should still be viewed and treated as wild animals.

Wildlife research within containment requires further consideration due to multiple factors including being restricted to indoor facilities, duration of the study, additional PPE, and other personnel constraints (e.g., advanced training) not typically required with other species or in other facilities. If the benefit of live wild animal research justifies the need to hold a species in captivity, a better understanding of the resources available to researchers will facilitate ethical and appropriate husbandry and handling. Raccoons and skunks are especially relevant North

American species due to their abundance in many environments and ecosystems, including urban and peri-domestic, and status as reservoirs for numerous zoonotic pathogens. As such, they are pivotal for investigations of pathogen dynamics and spillover risk investigations. We hope that the provided information will improve approaches to husbandry and handling methods of striped skunks (*Mephitis mephitis*) and northern raccoons (*Procyon lotor*) within research facilities as well as draw attention to the many challenges involved.

FIGURE LEGENDS AND TABLES

Figure 3.1 Modified modular kennels that housed both raccoons and skunks during the study (A). The dimension of each kennel was approximately 1.5x1.5x2.1 m and each kennel was fixed to an identical, adjacent kennel by an optional divider. The divider is depicted in the open position (B), but was closed for our study. The kennels were fully enclosed with 14-gauge wire fence panels and secured with outdoor nylon cable ties. The interior of each kennel was also reinforced with poultry fencing as depicted in (A), (B), and (C).

Figure 3.2 Raccoon and skunk cages in a Biosafety Level 3 Agriculture (BSL-3Ag) facility should be fully enclosed and equipped with denning areas (B), ample floorspace for food and water (A), substrate to climb (C), and various good-quality toys (A). Skunks while not known to climb, are capable if given appropriate substrate (D).

Figure 3.3 The raccoon on top of a small carrier, which acts as a den in this enclosure; note that the raccoon's right rump is clipped for identification purposes. As depicted in this figure, raccoons often are active, including interacting with their environment, especially when food is introduced, thus allowing ample opportunities for identification.

Figure 3.4 Image (A) is a demonstration of the technique that can be used to catch a skunk within its enclosure. Lightly grasp the base of the tail and slowly move your dominate hand over the shoulders of the animal to then "scruff" them. Image (B) depicts how you should "scruff" a skunk. Note that the handler is not lightly restraining the tail because the researcher is stabilizing the leg that they are injecting anesthetics into; in other cases, the tail would be lightly restrained to prevent adducting. A secure scruff hold will keep the researcher well out of research of a skunk's mouth, as well as nearly immobilize the animal's forelimbs. Image (B) is in an ABSL-2 facility, prior to BSL-3Ag transfer.

Figure 3.5 Image (A) is a demonstration of the technique that can be used to catch a raccoon that is loose in an enclosure. First, use your dominant hand to lightly pin the animal to the wall of the enclosure; then, use your other hand to either help control the head by securing it around the neck in conjunction with your dominant hand, or under the animal's rump to keep it from backing out of the hold. Image (B) depicts how you can securely restrain a raccoon while removing it from the wall; place one hand in the front of the thorax around the neck and shoulders and the other behind the thorax and neck to control the head. This raccoon is being restrained to receive oral medications in both cases. When releasing the animal, it is easiest to place them back on a wall.

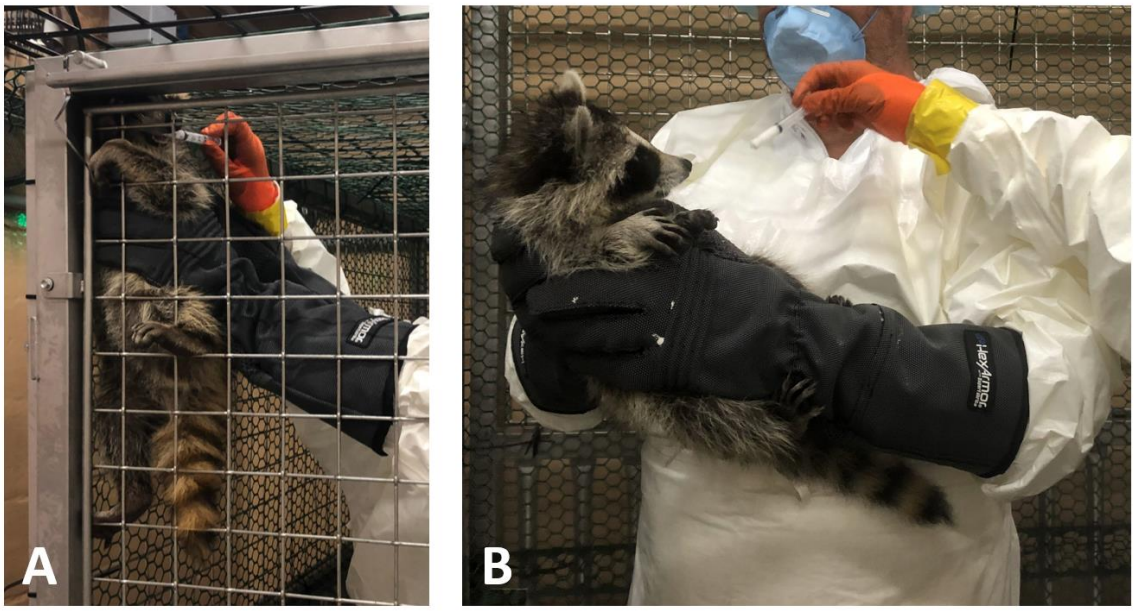

Figure 3.6 To facilitate sampling, a piece of plexiglass with a small hole was placed at chest height on the inside of the front wall of the kennel. Researchers would then place the animal's nose into the small hole in the Plexiglas and a nasal swab sample was taken. The plexiglass prevents the animal from grasping any of the cage bars, as depicted in image (B), while also not allowing the animal to bite the swab or fellow handler. Image (C) depicts an anesthetized raccoon; however, the circle made by the researcher's hands exemplifies how the panel in image (A) functions; ideally, the panel also covers the animal's eyes to keep it calm.

Table 3.1. Biologic parameters for both racoons and striped skunks.[10,27,39,44,51,57,60,63,64]

	Raccoons	Skunks
Weight (kg)	6-20	2-4
Temperature (F°)	95-102	98.6-102
Heart Rate (bpm)	94-134	140-190
Respiratory Rate (rpm)	20-30	35-40
Sexual Maturity (months)	12-24	10-12
Breeding Season	Jan.-March	Late winter-Early Spring
Estrus (days)	5-10	10
Gestation (days)	63	59-77
Litter Size	1-8	5-7
Eyes Open (weeks)	3-4	3-4
Wean (weeks)	7-8	6-8
Life Expectancy (years)	10-13	6-10

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