FULL TITLE

Navigating cross-reactivity and host species effects in a serological assay: A case study of the microscopic agglutination test for *Leptospira* serology

SHORT TITLE

Navigating cross-reactivity in a serological assay

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ROM, ACRG, BB, KCP and JOL-S conceived the study. RLG processed the biological samples. ROM, ACRG, and AHG created the figures and analyzed the data. ROM and ACRG drafted the manuscript. All authors revised the manuscript.

DATA ACCESSIBILITY

Code and data can be found at: https://github.com/rileymummah/x-reactivity/

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ABSTRACT

Background

Serology (the detection of antibodies formed by the host against an infecting pathogen) is frequently used to assess current infections and past exposure to specific pathogens. However, the presence of cross-reactivity among host antibodies in serological data makes it challenging to interpret the patterns and draw reliable conclusions about the infecting pathogen or strain.

Methodology/Principal Findings

In our study, we use microscopic agglutination test (MAT) serological data from three host species with confirmed infections to assess differences in cross-reactivity by host species and diagnostic lab. All host species are known to be infected with the same strain of *Leptospira interrogans*. We find that absolute and relative antibody titer magnitudes vary systematically across host species and diagnostic laboratories. Despite being infected by the same *Leptospira* serovar, three host species exhibit different cross-reactivity profiles to a 5-serovar diagnostic panel. We also observe that the cross-reactive antibody titer against a non-infecting serovar can remain detectable after the antibody titer against the infecting serovar declines below detectable levels.

Conclusions/Significance

Cross-reactivity in serological data makes interpretation difficult and can lead to common pitfalls. Our results show that the highest antibody titer is not a reliable indicator of infecting serovar and highlight

an intriguing role of host species in shaping reactivity patterns. On the other side, seronegativity against a given serovar does not rule out that serovar as the cause of infection. We show that titer magnitudes can be influenced by both host species and diagnostic laboratory, indicating that efforts to interpret absolute titer levels (e.g., as indicators of recent infection) must be calibrated to the system under study. Thus, we implore scientists and health officials using serological data for surveillance to interpret the data with caution.

AUTHOR SUMMARY

Serology is frequently used for disease surveillance, especially in systems that are resource constrained or logistically challenging. Serological testing involves analyzing blood serum samples to detect antibodies with reactivity toward specific pathogens (or more generally, molecular antigens), with the goal of characterizing past exposure to those pathogens. However, these antibodies can be non-specific and may react against other related pathogens or strains – a phenomenon known as cross-reactivity. Interpretation of serological data exhibiting cross-reactivity is difficult and simplifying assumptions are often made (e.g., to interpret the strain that elicits the highest antibody titer level as the infecting pathogen strain). Our work shows that interpreting antibody data requires more nuance and more caution. Both absolute titer levels and relative reactivity against different strains can vary across host species and diagnostic laboratory, so it is essential to interpret these data in the appropriate context. These host species differences in antibody reactivity and cross-reactivity patterns make direct comparisons across species inadvisable but may present an opportunity to use these patterns to learn more about circulating pathogen strains and transmission links in host communities.

INTRODUCTION

Identification of current infections and past exposure to specific pathogens is fundamental to studying the epidemiology and ecology of infectious diseases. The correct identification of the infecting species, serovar and/or strain is the basis for understanding intra- and interspecies epidemiological linkages. Serology, or the detection of serum antibodies formed by the host against an infecting pathogen, is used to detect individuals with current infections or prior exposure to a specific pathogen and is a widely used diagnostic for large-scale pathogen surveillance, particularly in wildlife systems.

Cross-reactivity among antibodies complicates serology-based surveillance of many pathogen groups including *Chlamydia* spp., *Shigella* spp., flavivruses, rickettsia, hantaviruses, *Salmonella* spp., and *Brucella* spp. (1–10,10–12). It is often assumed that the strain that elicits the highest antibody titer is the infecting strain, but titer magnitudes can depend on many factors including host species, host immune history, laboratory reference strains, or time since infection, so cross-reactions can distort this picture. Absolute titer levels are also used to estimate the recency of infection, but the quantitative titer dynamics (i.e., titer kinetics) among cross-reacting antibodies may differ such that the detected maximum titers and the rates of titer decline vary by strain (13–15). Thus, conclusions regarding the recency of infection for pathogens, such as *Leptospira, Salmonella,* or *Brucella,* whose serological tests assess antibody titers against a panel of infecting strains/serovars may differ depending on which antibody titer results are used. Additionally, when rates of decline differ among strains/serovars, seronegative results may also be unreliable. Antibody titers against the infecting strain could decline to undetectable levels while titers of cross-reacting antibodies against other strains may remain detectable. At the same time, characterization of cross-reactivity in serological testing could provide crucial insights into predictable relationships between cross-reacting antibodies, enabling accurate interpretation of serological results (16).

The microscopic agglutination test (MAT) is the serological diagnostic reference test for pathogenic species within the genus *Leptospira*, the causative agents of the disease leptospirosis (17).

The test consists of challenging serial dilutions of serum with live cultured bacteria and observing (with dark-field microscopy) the amount of agglutination that occurs due to serum antibodies binding to the antigen presented by the bacteria. An endpoint antibody titer is reported as the highest serum dilution that agglutinates at least half (50%) of the cells from the strain tested (18). However, diagnostic laboratories will often only test up to a specific serum dilution (yielding a a non-endpoint titer) because running a sample to titer endpoint can be labor- and cost-intensive. For *Leptospira,* MAT is typically performed using a panel of 1-20+ cultured isolates. Serovars (strains of *Leptospira* historically determined by serological reactions) are chosen for the panel based on what is known to circulate in the area or host species being tested. MAT is known to be affected by cross-reactivity and paradoxical reactions. Anti-*Leptospira* antibodies show a high degree of cross-reactivity in MAT results, whereby antibodies generated by infection with one strain will react with antigens of multiple strains (Chirathaworn et al., 2014; Smythe et al., 2009). Positive antibody titers against different strains make assessment of the infecting serovar and identification of epidemiological linkages difficult. Paradoxical MAT reactions, in which the early response is directed most strongly to a non-infecting serovar, are common in humans and other host species and further complicate any effort to identify the infecting strain from MAT results alone (14,15).

The cross agglutinin absorption test (CAAT) has traditionally been viewed as the gold standard isolate-based reference method for *Leptospira* serovar typing (18,21). While CAAT is used to identify, define, and describe potential new serovars, it is very rarely used for routine typing since it is a time-consuming method, and few reference labs are certified to perform it worldwide (22). Even with alternatives to CAAT such as pulsed-field gel electrophoresis (PFGE) and whole genome sequencing methods, *Leptospira* strain typing has traditionally required high concentrations of bacterial genetic material (22). Usually, the necessary quantity can only be obtained by growing the sampled isolate in specialized culture media. Growing a viable culture from a sample is time-consuming (on the scale of

120 months) and prone to failure, even when the sample is obtained from acutely ill animals (which are
121 assumed to have a higher bacterial load), so it is rarely performed. However if a cultured isolate can be
122 obtained, the strain can be typed reliably (or identified as a potential new strain) by PFGE or genome
123 sequence typing methods, which are much faster and cheaper alternatives to CAAT (23–25).

124 *Leptospira* genetics have revealed that the serological classification system does not match
125 genetic taxonomy. Historically, *Leptospira* was classified into serovars based on serological reactivity
126 and, furthermore, clustered into serogroups based on antigenically-related serovars (17). However, with
127 the dawn of genetic classification approaches, we learned that *Leptospira* serovars do not align neatly
128 with species delineations. Thus, serovars and serogroups can span multiple *Leptospira* species (e.g., *L.*
129 *interrogans* serovar Pomona and *L. kirschneri* serovar Mozdok belong to the same serogroup; Adler & de
130 la Peña Moctezuma, 2010; Arent et al., 2017). Furthermore, when this low resolution of serovar is
131 combined with potential cross-reactivity on serovar panels, there are clear advantages to using whole
132 genome sequencing when possible. Until recently, culturing *Leptospira* isolates was the limiting step in
133 reliable serovar identification; new genomic techniques have made it possible to acquire near-complete
134 genome sequences without an isolate (28) or identify serovar with genetic determinants (29), but these
135 can be cost-prohibitive.

136 In practice, many epidemiological and ecological studies of leptospirosis rely only on serum MAT
137 data due to its affordability, relative ease, and lack of reliance on obtaining isolates. MAT is recognized
138 as unreliable for strain typing because of cross-reactivity among serovars, but as it is often the only
139 evidence available, especially for wildlife systems, many authors use it as a basis to speculate on the
140 infecting serovar in their systems (e.g., Bishara et al., 2002; Panaphut et al., 2002; Santos et al., 2016;
141 Sehgal et al., 1995; Tunbridge et al., 2002). Unlike many commonly used serological tests, MAT does not
142 require host-specific reagents, which facilitates direct comparison between host species. This is
143 beneficial as many *Leptospira* serovars infect multiple mammal hosts. For example, *Leptospira*

interrogans serovar Pomona has been documented in deer, sea lions, pigs, island foxes, raccoons, coyotes, and striped skunks (13,35–39) to name a few. Correctly interpreting the differences and similarities in MAT results across different species is an important step in describing the ecology of *Leptospira* in a potential multi-host system. Whether the pattern of cross-reactivity against a specific serovar differs across host species has not been investigated or characterized.

In our study, we leverage a unique ecological system with one circulating strain of *Leptospira interrogans* in three sympatric wildlife host species and test the reliability of MAT as a tool to infer epidemiological processes. We specifically investigate the interpretation of maximum titers as markers of infecting serovar and the interpretation of quantitative titer levels as markers of time since exposure. We also highlight the potential confounding of host species and laboratory effects. Our results suggest that all MAT results (i.e., both absolute and relative quantitative titers) should be interpreted with caution and consideration of host species, while at the same time there is potential to infer powerful insights into infecting and circulating strains from host-serovar specific patterns of cross-reactivity.

DATA & METHODS

Study Animals and Sample Collection

Our dataset comprises samples from California sea lions (*Zalophus californianus*), island foxes (*Urocyon littoralis*), and island spotted skunks (*Spilogale gracilis*) with confirmed infections of *L. interrogans* serovar Pomona. Samples were collected from 107 sea lions that had stranded along the central California coast between 2004-2017 and were admitted to The Marine Mammal Center (TMMC; Sausalito, California) for rehabilitation. An additional thirty sea lion samples were collected from free-ranging wild sea lions from the central California coast and northern Oregon, between 2010 and 2012, as described in Prager et al., 2020. The majority of sea lions were diagnosed with acute leptospirosis (97/137) based on clinical signs, serum chemistry results, and necropsy data (41).

Samples from island foxes (n=59) and island spotted skunks (n=4) were collected between 2011 and 2016 during annual grid and target trapping conducted by the National Park Service (NPS) as part of a monitoring program on Santa Rosa Island, California. Santa Rosa Island has an area of approximately 214 km^2 and only three terrestrial mammal species (island foxes, island spotted skunks, and island deer mouse (*Peromyscus maniculatus*), and has no known history of *Leptospira* circulation before our study. Fox and sea lion data include both sexes and all age classes. All four skunks were adult males.

Ethics Statement

All California sea lion samples were collected under authority of Marine Mammal Protection Act Permits No. 932-1905-00/MA-009526 and No. 932-1489-10 issued by the National Marine Fisheries Service (NMFS), NMFS Permit Numbers 17115–03, 16087–03, and 13430. The sample collection protocol was approved by the Institutional Animal Care and Use Committees (IACUC) of The Marine Mammal Center (Sausalito, CA; protocol # 2008–3) and the University of California Los Angeles (ARC # 2012-035-12. UCLA is accredited by AAALAC International. The Marine Mammal Center and UCLA adhere to the national standards of the U.S. Public Health Service Policy on the Humane Care and Use of Laboratory Animals and the USDA Animal Welfare Act. Isoflurane gas was used to anesthetize all wild-caught, free-ranging sea lions for sampling. All island fox and skunk samples were collected by the National Park Service under USFWS permit TE-08267-2.

Sample Analysis

All animals included in this study had real time polymerase chain reaction (rt-PCR) confirmed *Leptospira* DNA in urine or kidney tissue as described by Wu et al (42), and the infecting *Leptospira* serovar was confirmed as *L. interrogans* serovar Pomona using PFGE as described previously by Galloway & Levett (23) on all cultured isolates (N_{CSL} = 19, N_{fox} = 11, N_{skunk} = 1).

Serum samples were tested by microagglutination test (MAT) against a panel of five *Leptospira* serovars comprising *L. interrogans* serovars Pomona, Autumnalis, Djasiman, Bratislava, and Icterohaemorrhagiae. Most of the samples included in this analysis were tested against more than five serovars (56 CSL samples and 7 fox samples were tested with a 20-serovar panel). We exclude tested serovars that yielded almost entirely negative or very low results for all host species, and serovars for which the overlap between tested samples was low among the host species. All titers used in the host species comparison were analyzed at the Centers for Disease Control and Prevention (CDC) in Atlanta, Georgia using MAT (as described in Prager et al., 2013) and run to endpoint dilution. Titer results were log-transformed for ease of interpretation using the following formula: $\log_2(\text{titer}/100) + 1$, thus a titer of 1:100 = 1, 1:200=2, 1:400=3, etc. Titers reported as <1:100 are represented by 0.

In a separate analysis focusing on variability among laboratories, a subset of 46 fox sera were MAT analyzed at three reference laboratories using a 2-serovar panel (Pomona and Autumnalis). The laboratories are referred to as Labs A, B, and C. Antibody titers against serovar Pomona were evaluated to endpoint at all three labs. Serovar Autumnalis was not titrated to endpoint for all samples at all labs. At Lab A, 43 of 46 samples were titrated to endpoint and 3 of 46 were only tested at a dilution of 1:100 (all were positive). At Lab B, all 46 samples were titrated to endpoint. At Lab C, all 46 serum samples were titrated to a 1:6400 dilution ($\log_2$ titer = 7) but not beyond.

Data Selection

To analyze antibody cross-reactivity patterns within and between host species, we selected MAT results from animals for which there was at least one positive urine PCR or culture result, which confirms current *Leptospira* infection. We did separate analyses for animals with PCR- or culture-confirmed infection ($n_{CSL} = 137$; $n_{fox} = 59$; $n_{sku} = 4$) and animals with confirmed infection and PFGE-confirmed serovar ($n_{CSL} = 19$; $n_{fox} = 11$; $n_{sku} = 4$). Only one skunk sample was PFGE-positive, so we

included samples from all four skunks in both analyses. We also performed an additional comparison of PCR- or culture-confirmed skunks with all MAT-positive skunks to confirm that patterns were consistent. For individuals that had been sampled longitudinally, we selected the MAT result from the serum sample with a collection date closest to that of the positive urine sample. The majority of MAT results from foxes (55/59) and all from skunks (4/4) were from sera collected on the same day as the *Leptospira* PCR- or culture-positive urine. Sea lion serum samples used for MAT were collected within 5 days of the date that the PCR- or culture-positive urine or kidney sample was collected (range = 0-5 days, median = 0 days). To analyze relative titer magnitudes among host species, we standardized antibody titer levels by dividing a given antibody titer by the highest antibody titer detected against any serovar in the 5-serovar MAT panel for that host serum sample.

We evaluated a subset of 46 fox serum samples at three certified testing laboratories as described above (see section on Sample Analysis) to compare MAT results across laboratories. Fox serum samples were chosen for this lab comparison based on MAT titer results from Lab A. For each MAT antibody titer level ranging from 1:100-1:51200, three serum samples with that MAT antibody titer against serovar Pomona, as reported by Lab A, were selected where possible (Table S1). In addition to these 30 samples, we included a further 10 samples that had no detectable antibodies against serovars Pomona and Autumnalis at Lab A, and six samples that had no detectable antibodies against serovar Pomona but were MAT positive against serovar Autumnalis at Lab A.

RESULTS

All host species exhibited strong antibody cross-reactivity against the five *Leptospira* serovars included in the MAT panel. The serovar against which the highest antibody titer was measured differed among the three host species, despite the fact that all were infected by *L. interrogans* serovar Pomona (Fig 1; Fig S1). The highest antibody titers detected in the majority of California sea lion (89.8%) and

spotted skunk (100%) samples were against serovar Pomona, but the highest antibody titer detected in Channel Island fox samples was most often against serovar Autumnalis (69.5%). Further, we detected a clear difference in the absolute magnitude of anti-*Leptospira* antibody titers across the three host species (Fig 2; Fig S2). Across four of the five serovars, sea lions exhibited consistently higher antibody titers relative to foxes and skunks. The exception was serovar Autumnalis, against which similar antibody titer magnitudes were detected in sea lions and foxes (Fig 2). Meanwhile, antibody titers detected in skunks were consistently lower than those from the other host species. Patterns were consistent between the PCR- and culture-confirmed dataset and the PFGE-confirmed dataset for all species (Figs S1 & S2). We further compared PCR- and culture-confirmed skunks to all skunks that were MAT-positive against one of the five serovars on the panel and found similar results (Fig S3).

We examined titer dynamics and changes in the cross-reactivity profile through the course of infection and recovery using individual-level longitudinal data from 46 foxes sampled from 2009-2019. In particular, one fox illustrated a course of infection during which the titer against the non-infecting serovar (Autumnalis) was always higher than the titer of the infecting serovar (Pomona) and remained positive after the latter declined to zero (Fig 3). Although this was the clearest case study of this phenomenon in our dataset, other individuals had similar courses of infection where their highest titer was consistently against a non-infecting serovar (Fig S4).

Analysis of 46 fox serum samples at three different diagnostic laboratories showed that both absolute and relative titer levels against serovars Pomona and Autumnalis varied systematically among labs (Fig 4). When comparing absolute antibody titer magnitude against serovar Pomona, the median titer was lowest from Lab A and highest from Lab C, with titers detected against serovar Pomona roughly one dilution greater at Lab B than Lab A, and more than three dilutions greater at Lab C than Lab A (Fig 4B). Endpoint titers against serovar Autumnalis were not run for all samples at all three laboratories so comparisons were not possible at greater than 1:6400 dilution ($\log_2$ titer = 7). Thirty-two of the samples

tested at Lab C were positive at dilutions less than 1:6400 against serovar Autumnalis, but endpoint titers for the 14 samples that were still positive at the 1:6400 dilution are unknown. When assessing relative titer magnitude between labs, we found that at Lab A, antibody titers against serovar Autumnalis were generally higher than those against serovar Pomona (Figs 4A & S5), whereas at Labs B and C, antibody titers detected against serovar Autumnalis were generally equal to (Lab B) or less than (Lab C) those against serovar Pomona (Figs 4A & S5)

DISCUSSION

We tested sera from three host species at three different testing laboratories using the MAT assay and found that antibody cross-reactivity patterns can differ qualitatively and quantitatively across host species, despite infection with the same causative agent (in our case study, the same species and serovar of *Leptospira*). We also showed that the highest detected antibody titer is not necessarily against the infecting serovar, and that both relative and absolute antibody titer magnitudes detected against different serovars can vary by diagnostic lab. MAT titers and cross-reactivity patterns are frequently used to characterize *Leptospira* epidemiology or ecology, with some studies proposing that the infecting serovar is that against which the highest MAT antibody titer is detected (30–34,43) or interpreting high MAT antibody titers against multiple serovars as proof of multiple circulating strains (44). Our results highlight that these interpretations are not robust and can lead to inaccurate conclusions regarding the epidemiology of *Leptospira* transmission dynamics within and between host species. This work raises clear caveats for the use and interpretation of MAT data, as well as questions regarding the biological mechanisms by which host species can influence MAT results. We outline lessons learned from our analyses and discuss the implications for interpreting MAT results.

Lesson 1: Highest titer does not always indicate infecting serovar.

In our study, antibody titers detected in sea lions and skunks were generally highest against serovar Pomona, while foxes typically had the highest titer against serovar Autumnalis (Figs 1, 2, S1 & S2), despite our genetic evidence showing that the infections were caused by serovar Pomona. These results highlight that the serovar against which the highest titer is detected should not be assumed to be the infecting serovar. Misidentification of the infecting serovar could result in a misunderstanding of multi-species transmission patterns with implications for disease management and control.

Lesson 2: Seronegativity must be interpreted with caution.

Our longitudinal samples show that antibody titers against the infecting serovar can decay below the level of detection before those against non-infecting serovars do. Thus, a seronegative result against a given serovar does not necessarily mean it was not the infecting serovar, even when juxtaposed with positive titers against other serovars. This phenomenon could lead to misclassifying the infecting serovar if we rely on MAT for strain identification, or mistakenly ruling out the serovar that caused the infection, especially if exposure occurred in the relatively distant past.

Lesson 3: Absolute and relative titer magnitudes depend on host species.

We observed significant differences in both absolute and relative MAT titer magnitudes among the three host species tested. The same infecting serovar of *Leptospira* gave rise to different MAT cross-reactivity profiles in different host species (Fig 1). In general, we see that sea lions have higher median titers than foxes, which in turn, have higher median titers than skunks across the five serovars (Fig 2). Autumnalis is a notable exception in which sea lions and foxes exhibit a similar median titer magnitude. The mechanisms underlying these differences are unknown.

Lesson 4: Absolute and relative titer magnitudes can differ across laboratories.

We observed systematic differences in absolute and relative titer magnitudes among three certified testing laboratories, including qualitative differences in which serovars elicited the highest titers from the same samples (Fig 4). Despite adherence to excellent laboratory standards and protocols, the nature of the MAT testing process means that some variation among labs is bound to exist. MAT is not standardized among labs, and variation both within and between labs is expected (45). Many factors which are difficult to control can contribute to the variation of MAT results, so caution is needed when comparing MAT titers across laboratories.

Implications for interpretation of MAT results

Of the more than 300 pathogenic *Leptospira* serovars currently described, most diagnostic MAT panels select a maximum of 20 serovars. In fact, cost and time restrictions typically limit panels to 4-6 serovars or fewer, particularly under conditions with fewer resources and lower testing capacity. This leaves the distinct possibility that a circulating serovar (and possibly the infecting serovar) could be omitted from the MAT panel, leading to potential for sub-optimal diagnostics and misunderstanding of circulating strains and transmission linkages. At the bottom line, it's important to recognize that the serovar associated with the maximum titer in a given panel is not necessarily the infecting strain.

Titer magnitudes are often used to assess active infections. However, given our finding of differences in absolute titer levels across host species, relying on titer thresholds inferred from data in one species to identify recent or active infections in another can lead to inaccurate diagnosis and poor incidence estimates. For example, longitudinally sampled sea lions acutely infected with *L. interrogans* serovar Pomona had initial $\log_2$ titers against serovar Pomona ranging from 10 to 12 and these titers declined with a half-life of around 17 days (40). Therefore if $\log_2$ titer thresholds used to define active infection in humans – 3 – or dogs – 4 – were applied to sea lions, many would be miscategorized as current infections (15,26); this could occur even if the infecting serovar was not included in the MAT

336 panel and the sea lion titers arose from antibody cross-reactivity. Our longitudinal fox data show that
337 foxes could be similarly miscategorized if the human or dog thresholds are applied to them, as some
338 foxes infected with *L. interrogans* serovar Pomona persist above the $\log_2 = 4$ threshold for years (Fig S3).
339 It is essential that any efforts to interpret absolute titer levels are calibrated to the system under study.
340 When this is done, titer magnitudes (and their decay) can be used to estimate the recency of infection
341 (13,46–48). Modern titer kinetics approaches have the potential to include additional host-specific
342 information about the relationship among serovars (i.e., MAT cross-reactivity profile) to estimate time
343 since infection and improve our understanding of when outbreaks may have occurred.

344 We know of no prior work showing host species differences in MAT profiles. These patterns may
345 be driven by different major histocompatibility complex (MHC) types and diversity (49,50), but more
346 work is needed to understand how immunogenetic differences among wildlife may impact serology. It is
347 noteworthy that the island fox population recently underwent a severe population bottleneck and
348 exhibits very low genetic diversity (and therefore MHC diversity; Robinson et al., 2016). Yet recent work
349 in coyotes in southern California revealed a similar pattern – with MAT titers against serovar Autumnalis
350 frequently exceeding those against serovar Pomona, despite known circulation of serovar Pomona in
351 coyotes – suggesting that this effect may occur more broadly among canids (37). Systematically
352 expanding surveillance across canid species and beyond could provide insights on the possible existence
353 of a host phylogenetic effect on MAT reactivity.

354 It is possible that some interspecies variation in titer magnitude was due to sampling bias. Over
355 two-thirds of sea lion samples were from animals experiencing acute leptospirosis – the disease caused
356 by *Leptospira* infection. By contrast, foxes and skunks were sampled during a routine trapping program
357 aimed at monitoring these sensitive populations, so sample collection was not biased by disease
358 severity. This could skew our observed antibody titers higher in the sea lions as their severe clinical
359 disease suggests a recent infection (52), but a modeling analysis of island fox titers estimated peak titers

against serovar Pomona of 6 to 9 $\log_2$ titers (13), consistent with values reported here for foxes, and lower than values reported for sea lions. It is clear that there is a large degree of immunological variability within and between species.

Variability in titer magnitudes has been documented across reference laboratories (53). The International Leptospirosis Society sponsors the annual International Proficiency Testing Scheme for the Leptospirosis MAT, intended to provide information on the quality of MAT testing and improve MAT testing performance worldwide (54). Early rounds of this program reported a wide variety of titer levels for the same sample and serovar (54). Although multifactorial, variation is probably driven chiefly by two main factors. First, MAT relies on live bacterial cultures, and there may be slight strain variations between labs and in different batches grown within a lab. For trustworthy MAT results, within-culture serovar identity must be verified regularly (40,54). Secondly, determining antibody titers by assessing agglutination under dark-field microscopy is subjective and requires significant expertise; even with best practices, some observer effect is inevitable. Altogether, many factors which are difficult to control can contribute to the variation of MAT results, so caution is needed when comparing MAT titers across laboratories.

Overall, overinterpretation of individual titer values can lead to misrepresentation of host relationships and circulating strains. Which begs the question, why use MAT at all? Genetic methods remain superior to serology for strain typing but are becoming more accessible, but serology has many benefits that are distinct from culture-based methods with respect to duration of positivity and the potential to learn from antibody titer kinetics (with appropriate interpretation). Despite issues with serological cross-reactivity, MAT is generally more affordable than culture-based methods, does not require specialized equipment, is often an easier sample to collect, and captures information on past infections. Given the broad accessibility and continuing worldwide use of this diagnostic, we need to interpret its results with appropriate caution, while capitalizing on all available information. There may

be an opportunity to improve assessment of the infecting serovar by exploiting consistent patterns in cross-reactivity against serovars within a host species, but more research is needed to describe these patterns within and across host species. The rising availability and falling cost of genetic methods, coupled with exciting new developments in obtaining whole genome sequences of *Leptospira* without culture isolates, point to a future where genetic typing adds clarity and certainty to *Leptospira* epidemiology and ecology.

Conclusions

Serology plays an irreplaceable role in infectious disease ecology and epidemiology, but cross-reactivity can lead to pitfalls in interpreting serological data to assess current and past exposure to specific pathogens. For our case study, we have shown that there can be substantial and consistent effects of host species that influence cross-reactivity profiles and quantitative titer levels, which could lead to erroneous conclusions about infecting serovars or recency of infection if appropriate caveats are not observed. This in turn can yield misleading interpretations about patterns of *Leptospira* circulation across host communities, or sources of zoonotic cases. This is especially true when relying on titer magnitude to determine infecting strain, or when samples have been analyzed at multiple laboratories. These findings have implications for all pathogen for which antibodies can cross-react with other species or strains, and we advise scientists and health officials using serological data for surveillance to interpret the data with suitable caution.

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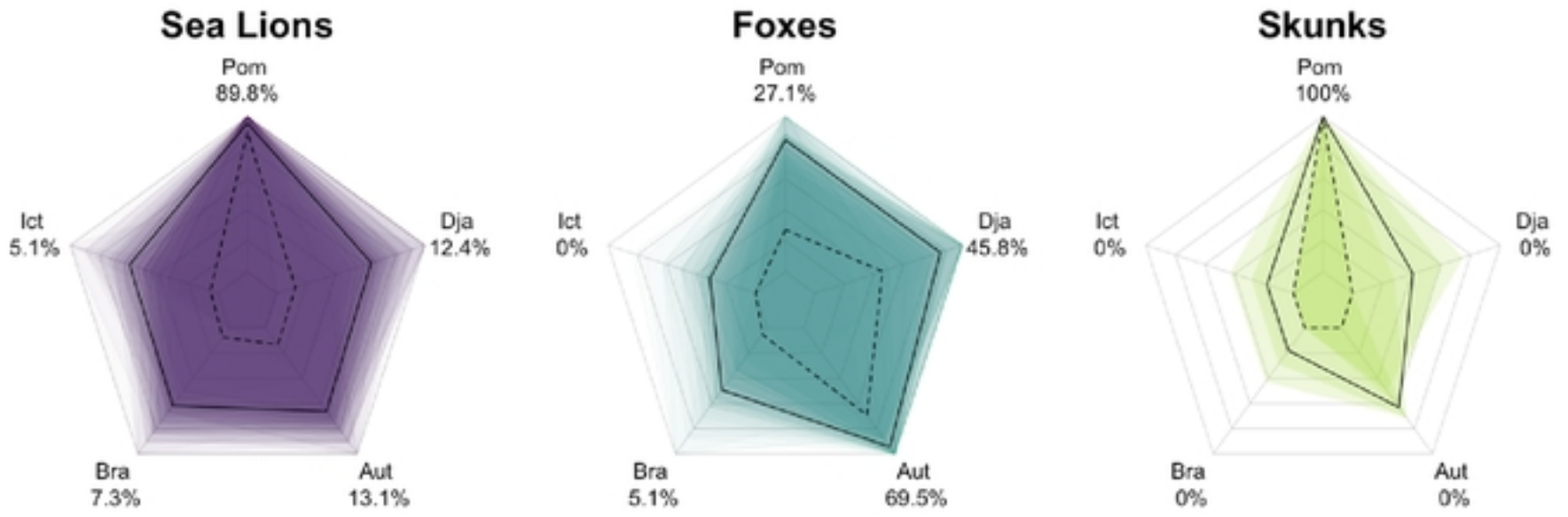

Figure 1. Host-specific patterns of relative MAT antibody titers detected against five *Leptospira* serovars (Pomona, Djasiman, Autumnalis, Bratislava, and Icterohaemorrhagiae) when the infecting serovar is *L. interrogans* serovar Pomona. Each plot shows the relative antibody titer levels (antibody titer against one serovar divided by the highest antibody titer detected against any serovar in the 5-serovar MAT panel run for that sample) for California sea lions (left; purple; n=56), island foxes (middle; cyan; n=56), and spotted skunks (right; green; n=4). The shaded regions on each plot are a representative subsample of overlaid polygons linking the values for an individual sample. The continuous black line shows the standardized antibody titer level for each sample (sample titer/maximum sample titer) averaged across all samples for each serovar for that species. The dashed black lines and the percentages associated with each serovar indicate the proportion of samples for which that serovar has the highest titer out of all serovars in that individual's panel, regardless of the actual titer. The numbers add up to more than 100% since multiple serovars can have the highest titer for any given sample (e.g., the highest antibody titer detected in the 5-serovar panel for that individual is both Pomona and Icterohaemorrhagiae, with titers of 1:6400).

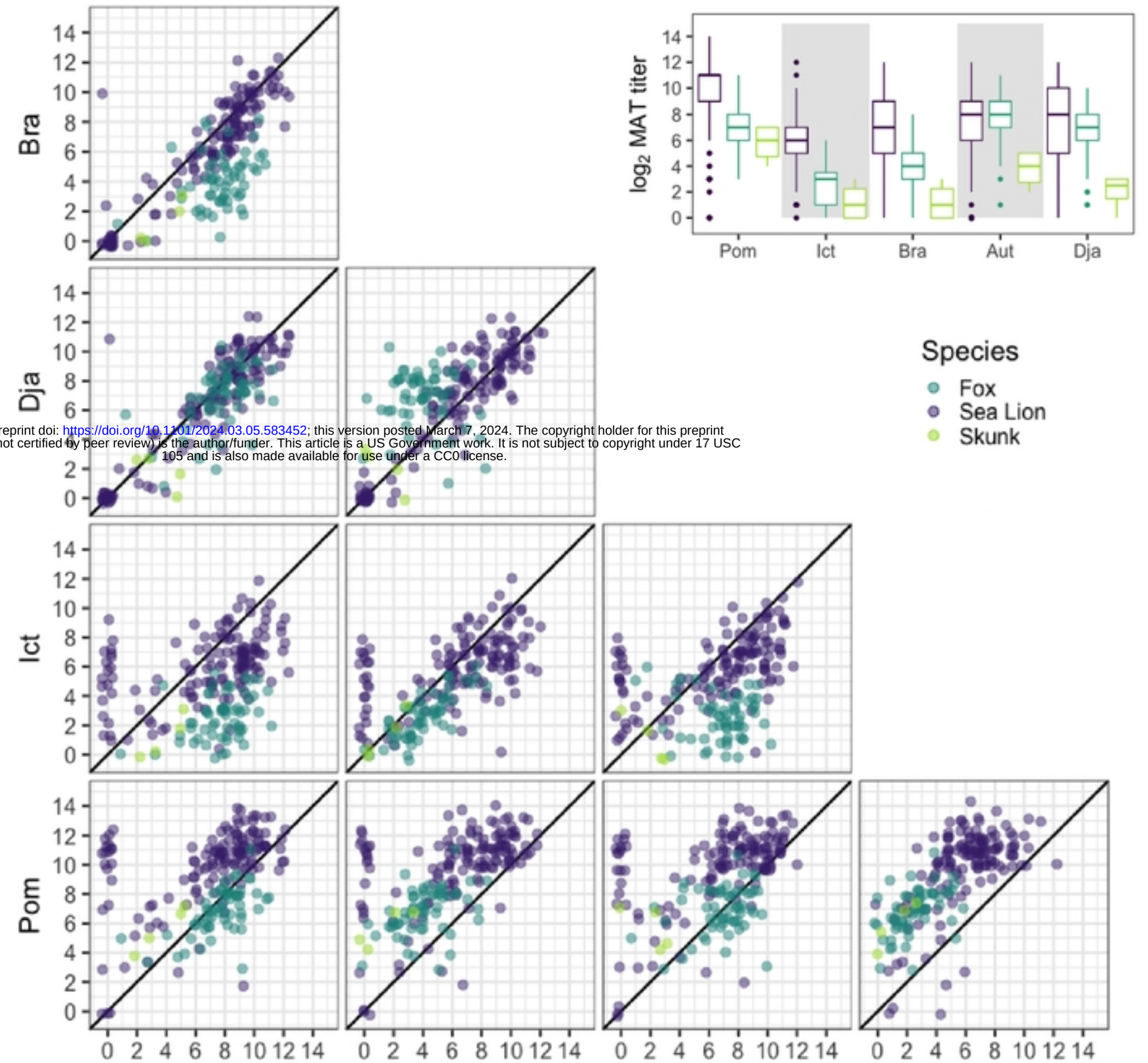

Figure 2. **Pairwise antibody titer levels against *Leptospira interrogans* serovars Pomona, Djasiman, Autumnalis, Bratislava, and Icterohaemorrhagiae in three host species.** Each plot shows the pairwise endpoint MAT titer levels (log_2 dilutions) for California sea lions (purple), Channel Island foxes (teal), and spotted skunks (green), all presumed to be infected with the same strain of serovar Pomona. The colors aggregate in a distinct pattern, showing that the serovar reactivity pattern is affected by the host species and that absolute titer magnitude differs among species. The black diagonal line corresponds to perfect

equivalence between different serovars. Jitter has been added to the points to aid visualization. Inset: differences in MAT titer magnitude against each serovar among host species.

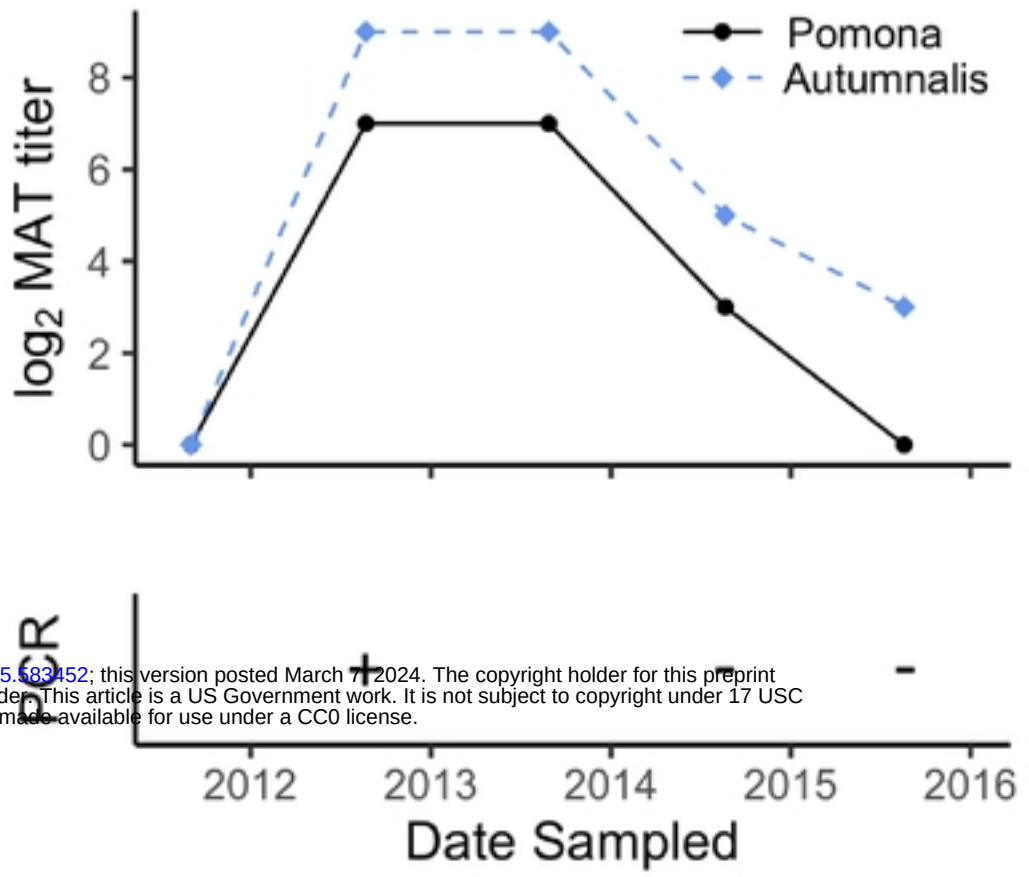

Figure 3. **Selected example of longitudinal antibody titer dynamics in a Channel Island fox**. The top panel shows antibody titers against *L. interrogans* serovars Pomona (black solid line) and Autumnalis (blue dashed line) from longitudinally collected serum samples from one fox. The bottom panel indicates the PCR test result from urine samples taken at the same time as serum collection.

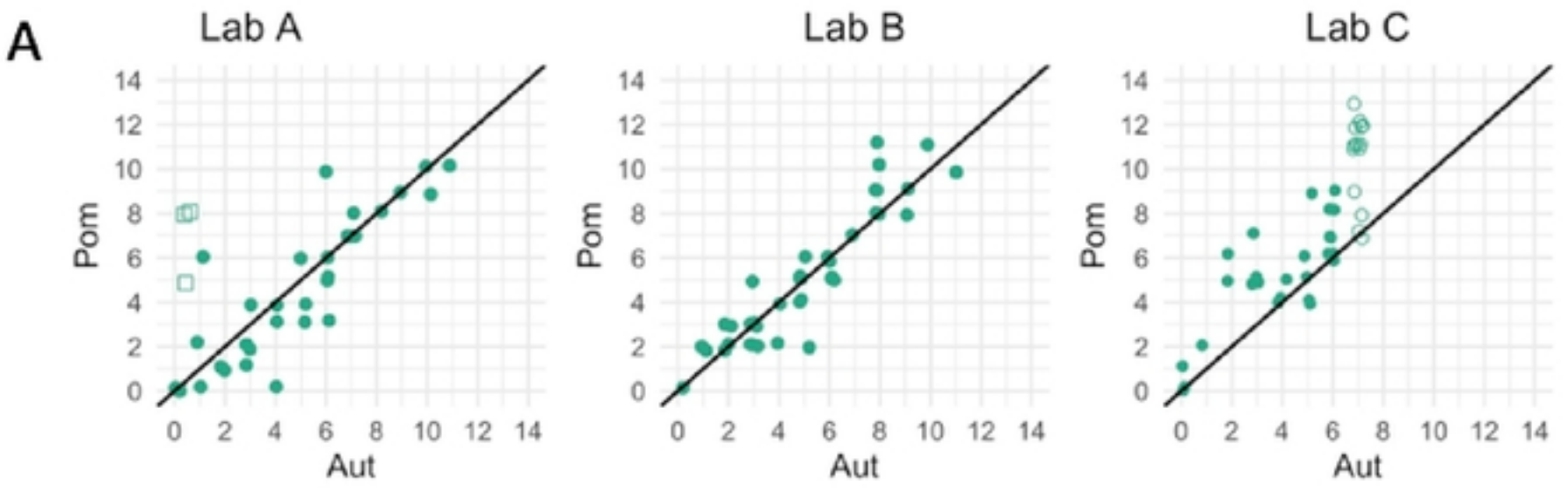

Figure 4. **Comparison of antibody titer results for fox serum samples evaluated at three testing labs.** Island fox serum samples (n=46) were tested in three different certified testing laboratories. The MAT antibody titers (log_2 dilutions) for serovars Pomona and Autumnalis are shown. All Pomona titers were run to endpoint dilution. In Panel A, open circles indicate non-endpoint Autumnalis titers at 1:6400 (log MAT titer 7) whereas open squares denote samples that were positive against serovar Autumnalis at 1:100, but no dilutions were performed. Jitter has been added to the points to aid visualization. Panel B represents the difference in antibody titer magnitude for a subset (n=32) of samples that were run to endpoint for serovars Autumnalis and Pomona at all three labs.