

1. REPORT NUMBER CA-20-3185	2. GOVERNMENT ASSOCIATION NUMBER	3. RECIPIENT'S CATALOG NUMBER
4. TITLE AND SUBTITLE Landscape Genomics Study on California Tiger Salamander		5. REPORT DATE July 2020
		6. PERFORMING ORGANIZATION CODE N/A
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9. PERFORMING ORGANIZATION NAME AND ADDRESS Department of Ecology and Evolutionary Biology, University of California, Los Angeles 10889 Wilshire Blvd., Suite 700 Los Angeles, CA 90095-1406		10. WORK UNIT NUMBER N/A
		11. CONTRACT OR GRANT NUMBER N/A
12. SPONSORING AGENCY AND ADDRESS California Department of Transportation (Caltrans) Division of Research, Innovation and System Information, MS-83 1727 30th Street Sacramento, CA 95816		13. TYPE OF REPORT AND PERIOD COVERED Final Report,
		14. SPONSORING AGENCY CODE N/A

15. SUPPLEMENTARY NOTES

16. ABSTRACT

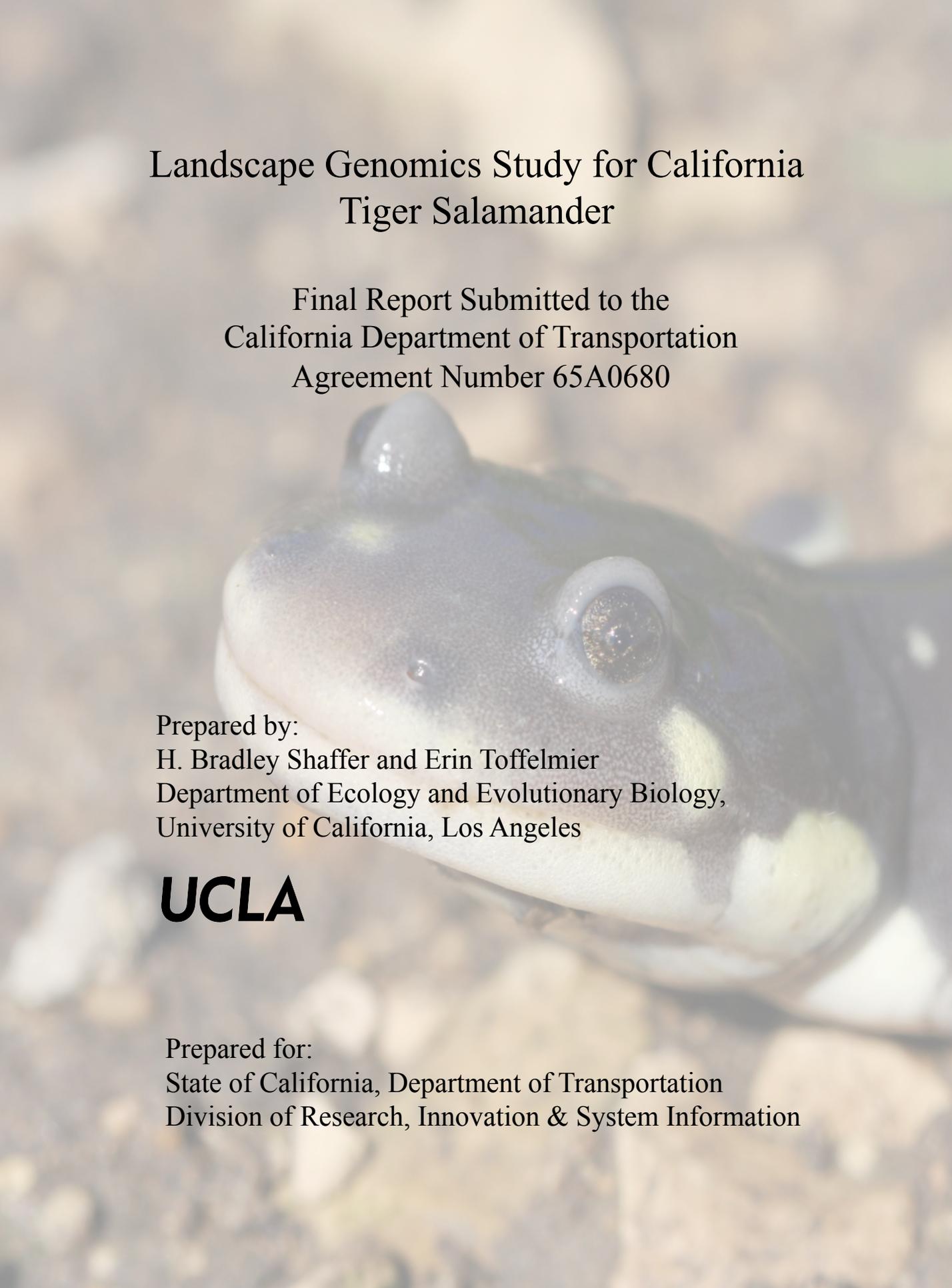
The California Tiger Salamander – *Ambystoma californiense* (CTS) is endemic to California. The Sonoma County distinct population segments (DPS) and Santa Barbara County DPS are federally listed as Endangered, while the Central California DPS is federally listed as Threatened. CTS is listed as Threatened under the California Endangered Species Act. Caltrans is required to consult with regulatory agencies about the impacts of transportation projects. A major conservation issue for CTS is the ongoing hybridization with the introduced non-native barred tiger salamander - *Ambystoma mavortium* (BTS). The two species hybridized in the areas where they came into contact creating admixed populations around the introduction sites and generating hybrid swarms. Thus, CTS recovery, in part, depends on preventing the geographical spread of introgression. Landscape genomics is at the leading edge of new genetic tools that provide fine scale, spatially explicit data allowing researchers and managers to achieve a deeper understanding of how different habitat types impede or enhance the resistance to movement of organisms on landscapes. This work constituted the first such genomic data set explicitly designed to quantify the effects of roads on gene movement in an endangered species. The most fundamental result of this study showed that, as originally predicted, roads appear to have a larger measurable effect on the movement of hybrid salamanders than on pure natives. Several possible mechanisms, detailed fully in the final report, may be responsible for this pattern, and we cannot disentangle them without explicit experiments that are beyond the scope of this project. However, it does appear that roads are a potent barrier to the movement of hybrid salamanders, and as such should be considered when planning for the movement of non-native genes in the future.

17. KEY WORDS California Tiger Salamander; landscape genomics, gene flow	18. DISTRIBUTION STATEMENT No Restriction.	
19. SECURITY CLASSIFICATION (of this report) Unclassified	20. NUMBER OF PAGES 29	21. COST OF REPORT CHARGED

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Landscape Genomics Study for California Tiger Salamander

Final Report Submitted to the
California Department of Transportation
Agreement Number 65A0680

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Landscape Genomics Study for California Tiger Salamander

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A Final Report Submitted to Caltrans, July 1, 2020

Introduction & Overview: The California Tiger Salamander, *Ambystoma californiense* (CTS) is a federally and state protected species that is endemic to California. The US Fish and Wildlife Service (USFWS) recognizes and individually protects three distinct population segments (DPSs) of CTS: Sonoma, Santa Barbara, and Central. CTS is also listed as Threatened under the California Endangered Species Act. The California Department of Transportation (Caltrans) is required to consult with regulatory agencies about the impacts of transportation projects on CTS during state and federal Endangered Species Act (ESA) consultations as well as during California Environmental Quality Act (CEQA) and National Environmental Policy Act (NEPA) review.

One of the primary management challenges for CTS centers on hybridization with the non-native barred tiger salamander, *Ambystoma mavortium* (BTS). In the 1950's thousands of BTS were imported as fishing bait to the Salinas Valley region of central California, and over the last 60 years they have extensively hybridized with CTS, producing viable hybrids that continue to expand and threaten the genetic and ecological integrity of protected CTS (Fitzpatrick and Shaffer, 2007; Fitzpatrick et al., 2010). Subsequent introductions and movements of BTS hybrids have spread this threat, including into the critically endangered Santa Barbara DPS. Laboratory research indicates that under many conditions hybrids are more active and move further and faster than CTS (Johnson et al. 2010). Understanding precisely how human structures, including roads, enhance or inhibit the movements of native and non-native genes, and whether roads may constitute an unrecognized tool to reduce the spread of non-native BTS genes, is now possible using genomic tools specifically developed for CTS (McCartney-Melstad et al, 2016).

The goals of this research are to quantify the effects of roads on the movements of native CTS and non-native BTS hybrids as they traverse natural landscapes. Previous work has shown that hybrid salamanders grow larger and move further under laboratory conditions (Johnson et al., 2010), suggesting that they may be more susceptible to road mortality than less mobile CTS. Understanding the effects of roads may be a key to slowing or stopping the movement of hybrids and therefore aiding recovery of native CTS. In addition, this research serves as a model for additional landscape genomic work on amphibians and reptiles that may also be impacted by roads. Given that western spadefoot (*Spea hammondi*), western pond turtle (*Emys (Actinemys) marmorata* and *Emys (Actinemys) pallida*), and foothill yellow-legged frog (*Rana boylei*) are candidates for federal and/or state listing, and several other migratory, declining amphibian and reptile species are already listed, using population genomic tools to assess the actual impacts of roads is an

important management tool for Caltrans.

We emphasize that although this is a final report, we are committed to additional data analyses and to formally publishing our results. In that sense, our analyses and conclusions are not complete. However, we are confident that we have addressed the goals of our project, and here present our data and results on the impact of roads as barriers to dispersal for native CTS and CTS-BTS hybrid populations across the landscape. **Our fundamental result is that, as originally predicted, roads appear to have a larger measurable effect on the movement of hybrid salamanders than on pure natives. Several possible mechanisms, detailed later in this report, may be responsible for this pattern, and we cannot disentangle them without explicit experiments that are beyond the scope of this project. However, it does appear that roads are a potent barrier to the movement of hybrid salamanders, and as such should be considered when planning for the movement of non-native genes in the future.**

Genetic Methods

DNA sequence generation

We generated sequence data from 5,237 exon regions for each sample using a target capture approach following McCartney-Melstad et al. (2018). We trimmed raw sequence data of adapters using skewer v0.2.2 (Jiang et al. 2014). We mapped target regions to the axolotl genome (Nowoshilow et al. 2018; Smith et al. 2019) using bwa mem (Li 2013) and retained targets that uniquely mapped to the genome. We then used the Genome Analysis Toolkit (GATK) version 3.8-1 to call single nucleotide polymorphisms (SNPs) and genotypes (DePristo et al. 2011; McKenna et al. 2010; Van der Auwera et al. 2013). We retained biallelic SNPs with $QD < 2.0$, $MQ < 20.0$, $FS > 60.0$, $MQRankSum < -12.5$, $-8.0 >$ $ReadPosRankSum > 8.0$, $SOR > 5.0$, and $QUAL < 30$. We phased genotypes using the paired-end read data with WhatsHap 0.17 (Martin et al. 2016; Patterson et al. 2015). This data set was used to estimate the hybrid index score (HIS, see below) for each pond sample. We then further filtered SNPs, allowing a maximum missing rate of 25% across all samples, minor allele frequency of greater than 0.005, and which were not singletons or private doubletons. We then selected SNPs with a maximum linkage disequilibrium correlation of 0.25 within 1000 base pair windows, and thinned remaining SNPs to less than one per 150 base pairs. This process yielded 7,359 SNPs across all samples.

Hybrid Index Scoring

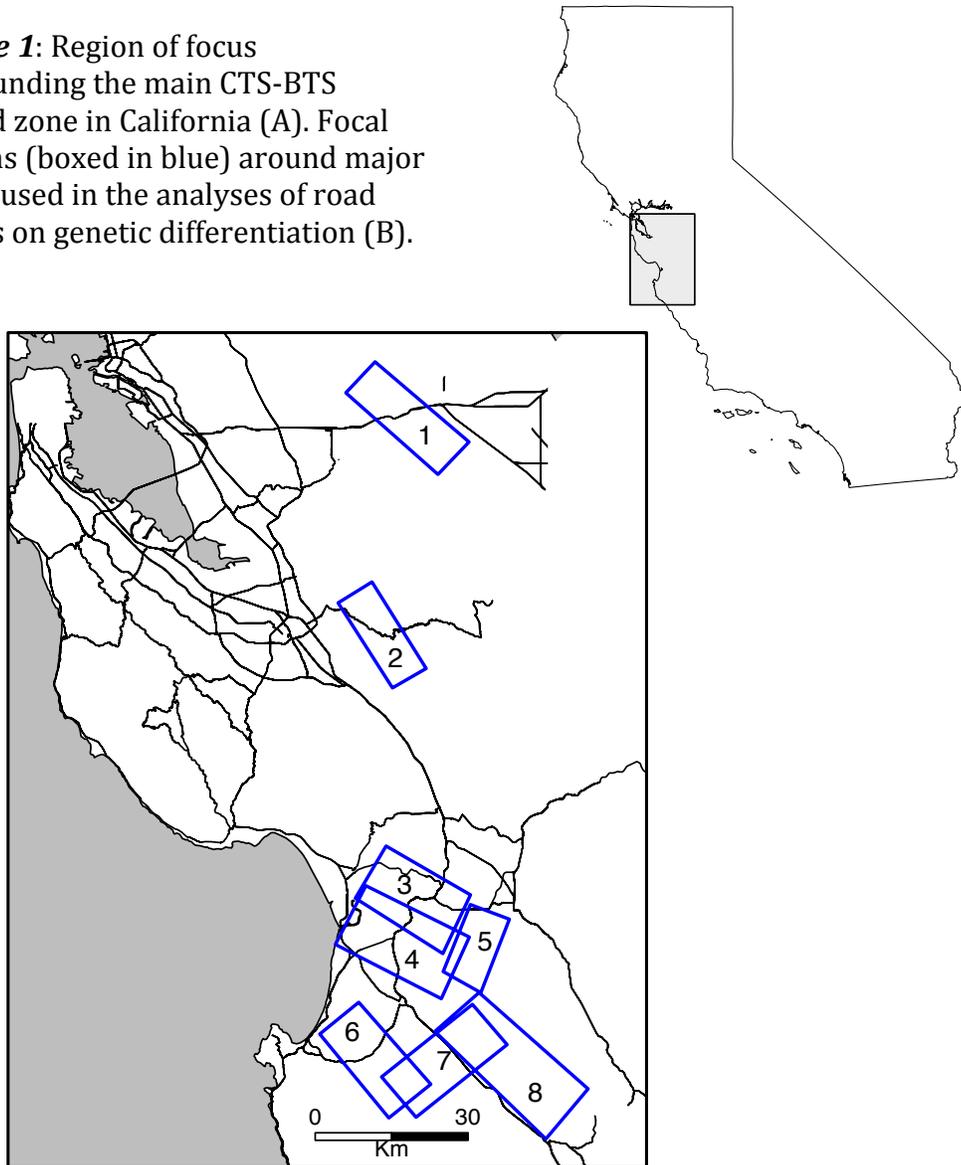
We used phylogenetic inference to quantify introduced ancestry, a new method to generate hybrid index scores under development by members of our lab group. Briefly, for each gene region, we sequenced reference samples known to be “pure” *Ambystoma californiense* (CTS), and *A. mavortium* (BTS), as well as sequences from other, unknown samples. After phasing all sequences, we generated a maximum likelihood phylogenetic tree for each locus (that is, each exon) with the GTR model of sequence evolution using FastTree 2.1.10 (Price, Dehal, and Arkin 2010). For each individual, we then scored both of their haplotypes as either BTS or CTS based on the patristic distance between the focal individual’s haplotype and the reference samples, yielding a genotype for each individual at each locus. We then calculated that individual’s hybrid index score as the average genotypic composition across

all loci scored for that individual. The per-pond HIS was calculated as the mean HIS across all individuals in that locality sample.

Spatial Analysis Methods

We used two complementary approaches to examine the impact of roads on hybrid and native ponds. We restricted these analyses to the main hybrid region centered around the Salinas Valley (Figure 1 inset) to exclude isolated hybrid ponds that are most likely recent human-mediated introductions and focus on hybrid ponds that are the product of the historical introductions followed by many generations of dispersal and migration. For each approach, we used samples from our existing collection, supplemented with the 14,057 tissue samples that we collected, with partial support from this grant, since Fall, 2017.

Figure 1: Region of focus surrounding the main CTS-BTS hybrid zone in California (A). Focal regions (boxed in blue) around major roads used in the analyses of road effects on genetic differentiation (B).



Road effects on pond Hybrid Index Score (HIS)

Here, we examined the relationship between the HIS of individual focal ponds and the road attributes of the landscape surrounding each pond. Our reasoning in doing these analyses is that if roads somehow select for more or less non-native populations, we should use that effect as a covariate in later analyses. In addition, if roads exert a strong differential effect on hybrid versus native tiger salamanders genotypes, we expected that the HIS and road density might point to such an effect. We examined road densities in upland regions surrounding focal breeding ponds for two biologically meaningful radii: 562m (the mean adult migration distance for CTS, Searcy and Shaffer 2011) and 2100m (the approximate distance maximum migration distance used by the USFWS, see USFWS, 2004; and Searcy and Shaffer, 2011). These distances represent the average and near-maximum distance, respectively, that an individual salamander might move in a year between breeding seasons. The landscape characteristics within these radii, including roads, are therefore those that a tiger salamander might reasonably encounter during migration. For each distance class, we selected a subset of relatively isolated focal ponds that were at least twice their distance class (562m, 2100m) from any other focal pond, and then created a buffer around each selected pond with that distance class radius. This yielded a subset of “independent” focal ponds with non-overlapping upland regions ($N = 86$ ponds in the 562 distance class (Figure 2A), and $N = 45$ ponds in the 2100m distance class (Figure 2C), utilizing 639 and 396 samples, respectively. We used this approach to avoid the non-independence that would result from measuring characteristics of shared upland habitat (although the two distance classes are not strictly independent from each other as most of the focal ponds in the 2100m subset are also represented in the 562m subset).

Within each distance class buffer area, we calculated the total surface area of roads. We obtained geospatial road layers for each county overlapping our study region (and for which we have sampled ponds: Merced, Monterey, San Benito, Santa Clara, Santa Cruz) from the US Census Bureau’s TIGER/Line database (US Census Bureau). We retained paved roads and assigned widths to each road type (see Table 1 for road descriptions and widths) to approximate the paved surface area of each road. We excluded dirt roads, walking paths, and other minor improvements since they presumably have limited or no impact on salamander mortality or movement (Table 1). Within each distance class, for each focal pond, we then calculated the total surface area of the roads that fell within that radius. We used the R packages raster v2.8-19 (Hijmans et al. 2019), sp v1.3-1 and rgdal v1.4-3 for all layer processing.

For each distance class, using all independent ponds, we used ANOVA to examine whether the presence or absence of BTS alleles in a focal pond was related to the road surface area around that pond. We then selected only ponds that contained hybrids and modeled focal pond HIS as a continuous response to the total surface area of roads around each pond, and the mean HIS of neighboring ponds using linear models. Neighbors for each distance class were defined as ponds that fell within the radius of that distance class. Finally, we compared HI scores among focal ponds that had neighbors to focal ponds without neighbors.

Table 1: Types of roads included in road area calculations. Adapted from the US Census Bureau.

MTFCC	Feature	Included/Excluded	Width (m)
S1100	Primary Road	Included	14.6
S1200	Secondary Road	Included	10.6
S1400	Local Neighborhood Road, Rural Road, City Street	Included	6
S1630	Ramp	Included	6
S1640	Service Drive usually along a limited access highway	Included	6
S1730	Alley	Included	6
S1780	Parking Lot Road	Included	6
S1500	Vehicular Trail (4WD)	Excluded	NA
S1710	Walkway/Pedestrian Trail	Excluded	NA
S1720	Stairway	Excluded	NA
S1740	Private Road for service vehicles (logging, oil fields, ranches, etc.)	Excluded	NA
S1750	Internal U.S. Census Bureau use	Excluded	NA
S1820	Bike Path or Trail	Excluded	NA
S1830	Bridle Path	Excluded	NA

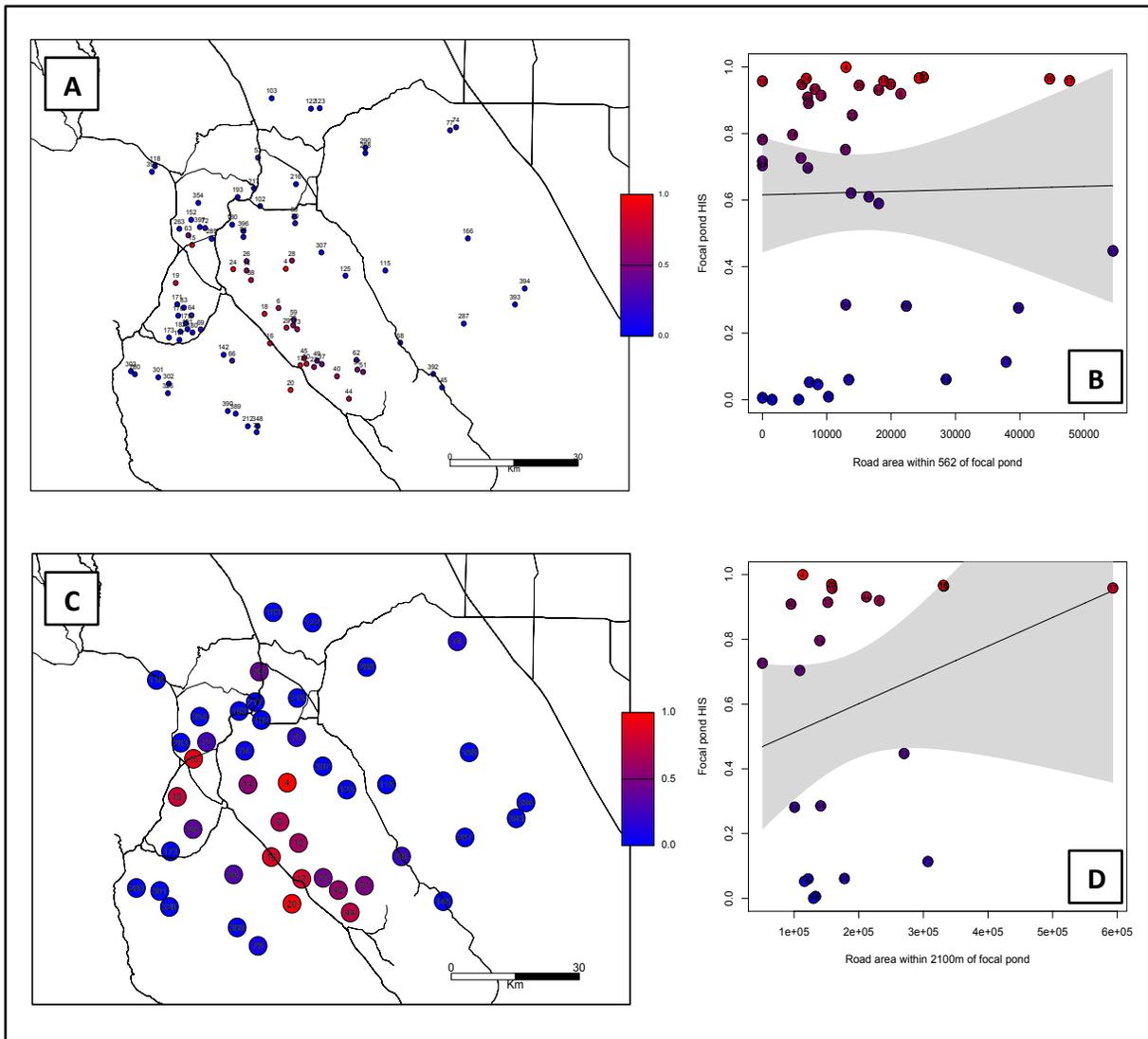


Figure 2: Map of pond localities with 562m buffer regions (A) and 2100m buffer regions (C). The relationships between road surface area and focal pond HIS were insignificant for both the 562m distance class (B) and the 2100m distance class (D). In all plots, individual ponds labeled with a unique numeric ID and are colored by their HIS with cool colors reflecting low HIS, and warm colors reflecting high HIS. The number next to each pond is a unique identifier. Black lines in panels A and C are major roads.

Road effects on genetic differentiation

We next examined whether the presence of intervening major roads impacts genetic differentiation between ponds. Here, the goal was to ask whether 1) roads affect genetic differentiation between ponds (we presume that they lower gene flow and so reduce population connectivity), and 2) whether any measured effect of roads was different for pure CTS and hybrid populations. We replicated this analysis across eight focal regions (seven regions centered across major highways and one additional region with no major highways

and secondary roads, see Figure 1B for the placement of these regions, Supplemental Figures S1-8 for details of each, and Table 2 for a summary of the number of ponds in each).

Table 2: The number of localities in each region used in analyses of pairwise genetic distances. The total number of unique ponds (147) in the concatenated data set used to build the MLPE models is less than the sum across all regions because some ponds are in two regions where the regions overlap.

Region Number	Road Name	Total Number of Ponds in Region	Number of Ponds with BTS Alleles	Number of Pure CTS Ponds
1	I-580	27	0	27
2	CA-130	22	1	21
3	US-101 - San Juan Bautista	9	1	8
4	US-101 - Salinas	16	8	8
5	San Juan Canyon Road	9	8	1
6	CA-68	33	5	28
7	US-101 - Salinas Valley	16	13	3
8	Salinas Valley - No Major Roads	34	34	0

For each pair of populations within each region, we classified each pair of ponds as 1) on the same side or different sides of the focal road and 2) whether non-native BTS alleles were present in both, one, or neither of the ponds in each pair. We generated estimates of genetic distance (linearized F_{ST}) for all population pairs within each region based on Weir and Cockerham's (1984) theta, calculated in the R package *assigner* v0.5.7. We calculated pairwise geographic distance as the great-circle straight line distance between the centroids of each pond in the pair with the package *fields* v9.7 (Nychka et al. 2017). We pooled all comparisons, and because some regions overlap, we removed replicates of comparisons that were represented more than once. This resulted in 613 CTS to CTS same side of a road (C-C-same) comparisons, 380 CTS to CTS on different sides of a road (C-C-diff), 129 hybrid to CTS on the same side of a road (H-C-same), 142 hybrid to CTS on different sides of a road (H-C-diff), 594 hybrid to hybrid on the same side of a road (H-H-same), and 66 hybrid to hybrid on different side of a road (H-H-diff) comparisons. These comparisons were based on 1210 individual genotyped samples across 147 unique localities. We fit linear mixed effects models with maximum likelihood parameter estimation (MLPE) and modeled genetic distance between two ponds as a function of 1) geographic distance, 2) road presence/absence between ponds, and 3) the presence of BTS alleles in one, both, or neither pond in the pair. MPLE models account for non-independence of pairwise data (in this case, a single pond may be compared to multiple other ponds) by specifying a covariate structure that accounts for non-independence in the pairwise matrix (Clarke, Rothery, and Raybould 2002; Shirk, Landguth, and Cushman 2017). We generated a full model with all variables plus six sub-models for all combinations of the three predictors (geographic distance, road side and genotypic comparison) (Table 3), and describe model fit as R^2_{β} , which works well

for models in which residual maximum-likelihood parameter estimation is used (Edwards et al. 2008).

Results

Road effects on pond hybrid index score of focal pond

For both 562m and 2100m distance classes, we found no significant relationship (562m: $F=0.01369$, $p=0.9075$; 2100m: $F=1.52$, $p=0.232$) between focal pond HIS and road surface area within that distance (Figure 2B and Figure 2D). For ponds with neighbors, HIS was positively related to the mean HIS of neighboring ponds in both distance classes (562m: $F=289.2$, $p<0.001$; 2100m: $F=115.2$, $p<0.001$), indicating that ponds within migration-distance proximity to each other tend to have similar, or spatially autocorrelated HISs. There was no difference in HIS between ponds with or without any neighbors within 562m ($F=0.618$, $p=0.434$); this could not be examined at the larger distance class because all focal ponds had at least 1 neighbor within 2100m. There was no difference in total road surface area between ponds with or without hybrids in either distance class (562m: $F=0.001$, $p=0.981$, 2100m: $F=0.007$, $p=0.934$).

In summary, our results indicate that the presence, or density of roads within the single-season migratory distance of tiger salamanders has no measurable effect on the HIS of focal ponds.

Road effects on genetic differentiation

Model rankings and coefficients are provided in Table 3. The highest ranking model ($R^2_{\beta}=0.357$) explaining variation in the pairwise genetic differentiation between ponds was one that include geographic distance between ponds and genetic comparison (i.e. did not include side of road), followed by genetic comparison only ($R^2_{\beta}=0.319$), and the full model ($R^2_{\beta}=0.251$). However, this does not mean that roads are not a barrier to gene flow, but only that they are not as important as the other two factors. To examine the effect of roads, we focus these results and subsequent discussion on the full model, which includes all three factors. In the full model, we found an overall trend of isolation by distance (slope = $0.008F_{st}/km$), as well as evidence for weak, but always positive isolation by distance for each possible combination of road (same or different side) and genotype class. (Plots for each type of comparison are provided in Supplemental Figure S9.) This is to be expected, and simply emphasizes that nearby ponds tend to be more similar genetically than more distant ones, even over the small spatial scales (1-25 km) in this analysis.

Table 3: MLPE model rankings and coefficients. We focus our discussion on the Full model, which includes geographic distance, roadside, and type of genetic comparison. For each variable in each model, we report the model coefficient first followed by the associated p -value. A “-” indicates that variable was not included in the model.

Model	R^2_β	<i>Int.</i>	Geographic Distance	Same Side (ref = Diff Side)	HYB-CTS (ref = HYB-HYB)	CTS-CTS (ref = HYB-HYB)	Same Side: HYB-CTS	Same Side: CTS-CTS
distance + genetic comparison	0.357	0.168 <0.001	0.008 <0.001	-	0.110 <0.001	-0.060 0.146	-	-
genetic comparison	0.319	0.232 <0.001	-	-	0.116 <0.001	-0.068 0.111	-	-
Full	0.251	0.272 <0.001	0.008 <0.001	-0.145 <0.001	0.006 0.831	-0.173 <0.001	0.197 <0.001	0.156 <0.001
side of road + genetic comparison	0.219	0.375 <0.001	-	-0.192 <0.001	0.012 0.659	-0.180 <0.001	0.180 <0.001	0.140 <0.001
distance	0.076	0.148 <0.001	0.008 <0.001	-	-	-	-	-
distance + side of road	0.076	0.149 <0.001	0.008 <0.001	-0.001 <0.001	-	-	-	-
side of road	0.032	0.255 <0.001	-	-0.065 <0.001	-	-	-	-

The most important comparisons showing the average effect of roads are presented in Figure 3. Figure 3A and 3B show, at the same scale, C-C and H-H differences across roads (panel 3A) and C-C and H-H on the same side of roads (panel 3B). Hybrid to hybrid pond comparisons exhibit higher pairwise F_{ST} values than C-C comparisons on different sides of roads (Figure 3A), but similar F_{ST} values in comparisons made on the same side of the road (Figure 3B). Figure 3C and 3D break these data down differently, and shows the comparison of C-C (panel 3C) and H-H (panel 3D) ponds on the same and different sides of roads pooled across our study areas. F_{ST} values are similar in CTS-CTS comparisons on same versus different side of the road (Figure 3C), suggesting that there is little measurable effect of roads on pure CTS population differentiation (the two lines are virtually indistinguishable). However, H-H comparisons on different sides of the road are higher than the same comparisons on the same side of the road (Figure 3D), suggesting a stronger impact of roads on hybrid genotypes. F_{ST} between CTS-hybrid comparisons are high in comparisons made on both the same and different sides of roads (Supplemental 10A,B), and do not show evidence of differential road effects (Supplemental Figure S10C).

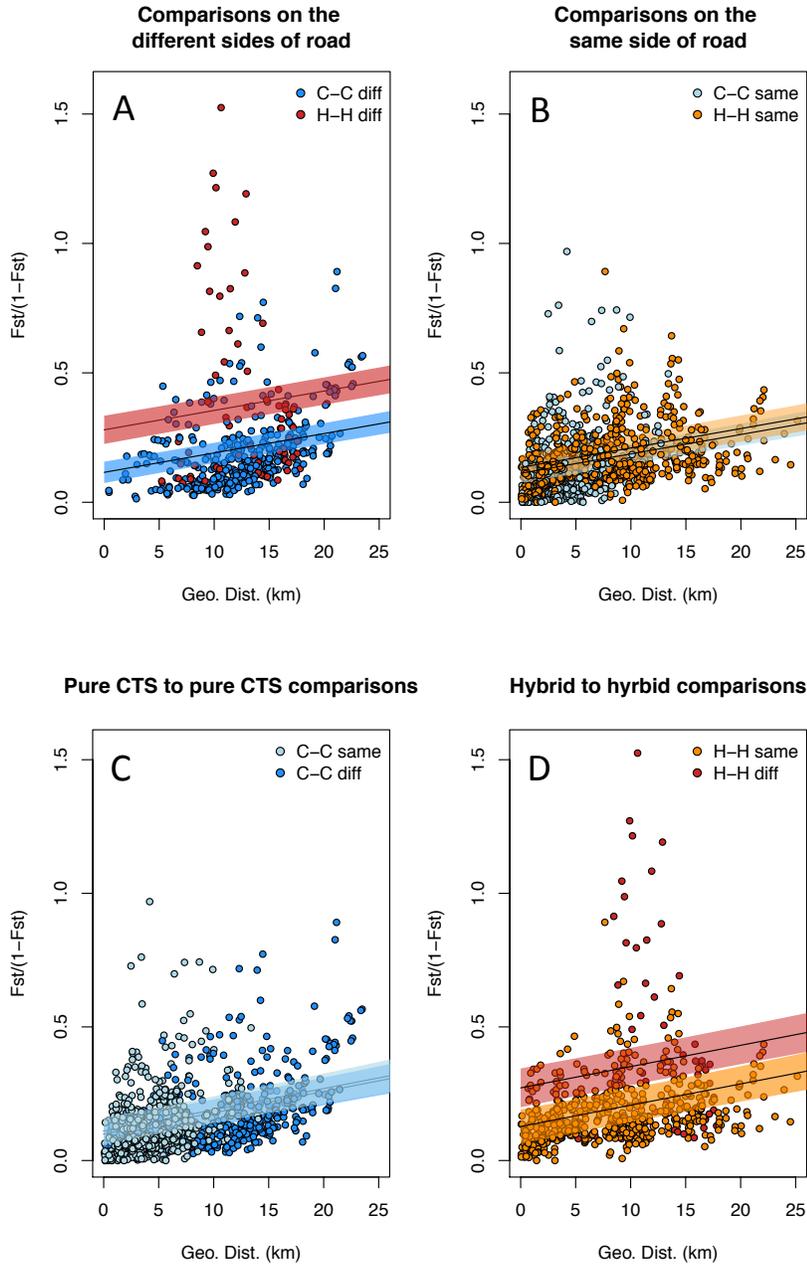


Figure 3: Genetic differentiation is higher between pure CTS ponds (CTS-CTS, “C-C”) than between hybrid ponds (hybrid-hybrid, “H-H”) when the two ponds are on different sides of a road (A), but differentiation within these two categories (C-C and H-H) is similar when the pair of ponds in the comparison are on the same side of a road (B). Differentiation in CTS-CTS comparisons is similar on the same and different sides of roads (C), while hybrid-hybrid comparisons exhibit higher differentiation when the two ponds are on different sides of a road (D). In each panel, each point represents a comparison between two ponds, the black lines represent the model for each comparison category, and the shaded region around each line is the 95% confidence interval for that model, colored by the comparison category.

Discussion

Roads are a strong, often negative landscape component with respect to wildlife conservation and management. For reptiles and amphibians, a recent synopsis of 166 California species found that most taxa experience Very High, High, or Medium demographic risk from roads; relatively few fell in the Low and Very Low risk categories (Brehme et al., 2018). A similar recent analysis focused on the threatened Western pond turtle complex (*Emys marmorata*, *E. pallida*), found a similar result—the higher the density of roads, the greater was the likelihood that a population would be male biased, presumably due to road mortality of nesting females (Nicholson et al., 2020). These results confirm any field biologist’s common-sense conclusions—animals die on roads, and the more roads in a given area, the greater the impact. For slow-moving, highly migratory species like tiger salamanders, the risk from vehicle-associated road mortality must be quite high. Brehme et al. (2018) placed California tiger salamander (CTS) in their highest risk category, based entirely on terrestrial risk associated with crossing roads. As pointed out by Brehme et al. (2018), “road mortality and habitat fragmentation are primary threats to the California tiger salamander and other Ambystomid salamanders because terrestrial habitat is used for interpond migration and overwintering”(p. 930).

However, in the relatively uncommon cases where a conservation goal involves eliminating non-native or hybrid individuals from a complex landscape, high road mortality may actually be a conservation tool worth considering. Our goals for this project were focused on developing the most robust genetic data possible to measure the impacts of roads on the movement of two classes of California tiger salamander. The first is protected under state and federal law—these are pure populations of endangered and threatened *Ambystoma californiense* (CTS). The second is a broad class of hybrid individuals between invasive barred tiger salamanders (*Ambystoma mavortium*, BTS) and native CTS. CTS are a declining, protected species. Both BTS and hybrids resulting from BTS-CTS admixture are viewed as a threat to CTS, and ideally would be eliminated and replaced by CTS. An important conservation goal is to stop, and ultimately reverse, the spread of BTS genes into CTS populations. Our goal with this research was to ask whether roads differentially inhibit the movement of hybrids compared to CTS. If so, then in areas where hybrids predominate, roads and their associated mortality may actually make a positive contribution to conservation outcomes of this threatened salamander.

Our primary results center on the differential effects of roads, as quantified by F_{ST} , between CTS and hybrid pond pairs on the same and different sides of major roads. As shown in Figure 3B and the Full model in Table 3, there is a clear pattern of isolation by distance between both CTS-CTS and hybrid-hybrid pond comparisons on the same side of roads, and this effect is roughly equal for the two genetic classes of salamanders. This makes sense—extensive field work has shown for native CTS that they move up to a maximum of 2000 meters within a breeding season (Searcy and Shaffer 2011; USFWS, 2004) and that there is often subtle but discernable genetic differentiation among nearby ponds (Wang et al., 2009; Wang and Shaffer, 2017). Although this has not been previously studied in CTS-BTS

hybrids, other members of the tiger salamander complex show similar levels of genetic differentiation over distances of tens of kilometers (McCartney-Melstad et al., 2018), and the data presented here demonstrate that hybrids show a virtually identical effect of isolation by distance in roadless comparisons (Figure 3B, and Supplemental Figure S9).

The difference in the effect of roads becomes clear when we examine genetic differentiation, corrected for geographic distance, between ponds on opposite sides of roads (Figure 3A, Table 3). Here, the difference is striking—average genetic differentiation is twice as great between hybrid pond pairs compared to CTS pond pairs across roads. Another way to visualize this difference is by comparing the insignificant genetic differentiation between CTS ponds on the same and different sides of roads (Figure 3C) to the striking difference for hybrid-hybrid ponds (Figure 3D). The result is clear—roads affect hybrid pond genomic differentiation more than they do pure CTS ponds. This suggests that, in principle, roads may have relatively little impact on CTS gene flow compared to their effects on hybrid gene flow. Roads therefore appear to be a potential tool to use in reducing gene flow, and limiting the spread of non-native genes, in this system.

The question that remains is why this result obtains, and what it says about the actual biological impact of roads on CTS compared to hybrid movement and gene flow. We can see at least three possibilities.

Hypothesis 1: Hybrids unsuccessfully cross busy roads more frequently than CTS, leading to greater mortality and increased genetic divergence in hybrids. This is the most straightforward explanation, and it may well be correct. Given that hybrid and CTS isolation by distance is virtually identical in the absence of roads (Figure 3B), this interpretation could be driven by several factors. One is that hybrids may migrate earlier in the night, or under less intense rain conditions, when more traffic, and therefore greater road mortality conditions occur than for CTS. Recent work (Carter et al., in submission) suggests that hybrids are more physiologically active than CTS, and that higher basal metabolic rate may mean that they are more likely to move earlier when cars are on the roads. Under this or related interpretations, our recommendation is to let cars proceed unimpeded in areas where hybrid salamanders are active, and embrace higher hybrid road mortality as a positive conservation outcome.

Hypothesis 2: The greater differentiation seen in hybrids compared to CTS is a function of BTS introduction history 60 years ago. We know a fair amount about introduction histories of BTS (Fitzpatrick and Shaffer 2007), and it is possible that non-native BTS were largely introduced only on one side of a given road. If a very few individuals have managed to cross the road, then the sampling variance associated with that very small propagule of BTS genes might drive the differentiation across roads (or even the lack of hybrids on one side of a road compared to the other). In this case, the difference in F_{ST} in hybrids compared to CTS is less due to direct road mortality than to the reticence of hybrids to cross roads. However, from a practical standpoint, the result is the same—roads stop hybrids from extensively migrating. Once again, roads should help prevent the spread of non-native genes.

Hypothesis 3: Some other artifacts of introduction history are responsible for the greater apparent impact of roads on hybrids compared to CTS. There are other possibilities—for example, perhaps introductions from different native BTS stocks (i.e. BTS translocated from different source regions into different introduction sites; see Johnson et al., 2011 for a history of BTS introductions across California) were deposited on different sides of roads. Although we have no evidence for this, it is possible, and it could enhance the genetic differentiation across roads. However, even in this unlikely scenario, it implies that hybrids are not successfully crossing roads with high frequency—if they were, then these differences would not be maintained by roads. Again, the message seems clear—roads inhibit the movement of hybrid salamanders.

Next steps and final conclusions

Our results are purely correlational—we examined patterns of differentiation and are attempting to infer causality from those patterns. That is always dangerous, and one must proceed cautiously. However, our strong conclusion from these correlational analyses are clear—roads appear to inhibit gene flow between hybrid compared to native CTS ponds in the central coast region of California. Obviously, we would like to see experiments that more directly confirm or refute these results, and they are possible. Radio transmitter data on hybrids and pure CTS could quantify movement patterns with respect to roads, or more extensive sequencing of long blocks of contiguous DNA from animals on both sides of a road can quantify the number of full sibs/close relatives that have crossed a road for CTS and hybrid genomic classes. Our prediction is that fewer hybrids will be found to have successfully crossed roads, but only additional data can confirm that prediction. In the meantime, our results indicate that roads are a potentially powerful conservation tool that can be used to limit population movement in hybrid swarms where we seek to slow or stop the spread of non-native genes. To take just one isolated example: in the Santa Barbara distinct population segment, non-native salamanders have been confirmed to exist immediately south of State Highway 246. There are many native CTS populations north of the highway, but based on our sequence analysis of over 400 individuals spanning 20 years of collecting, BTS genes have not yet crossed the highway. In this case, the Purisima recovery metapopulation may be protected from non-native BTS genes by Highway 246.

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Supplemental Figures

Region 1: I-580
All pure CTS

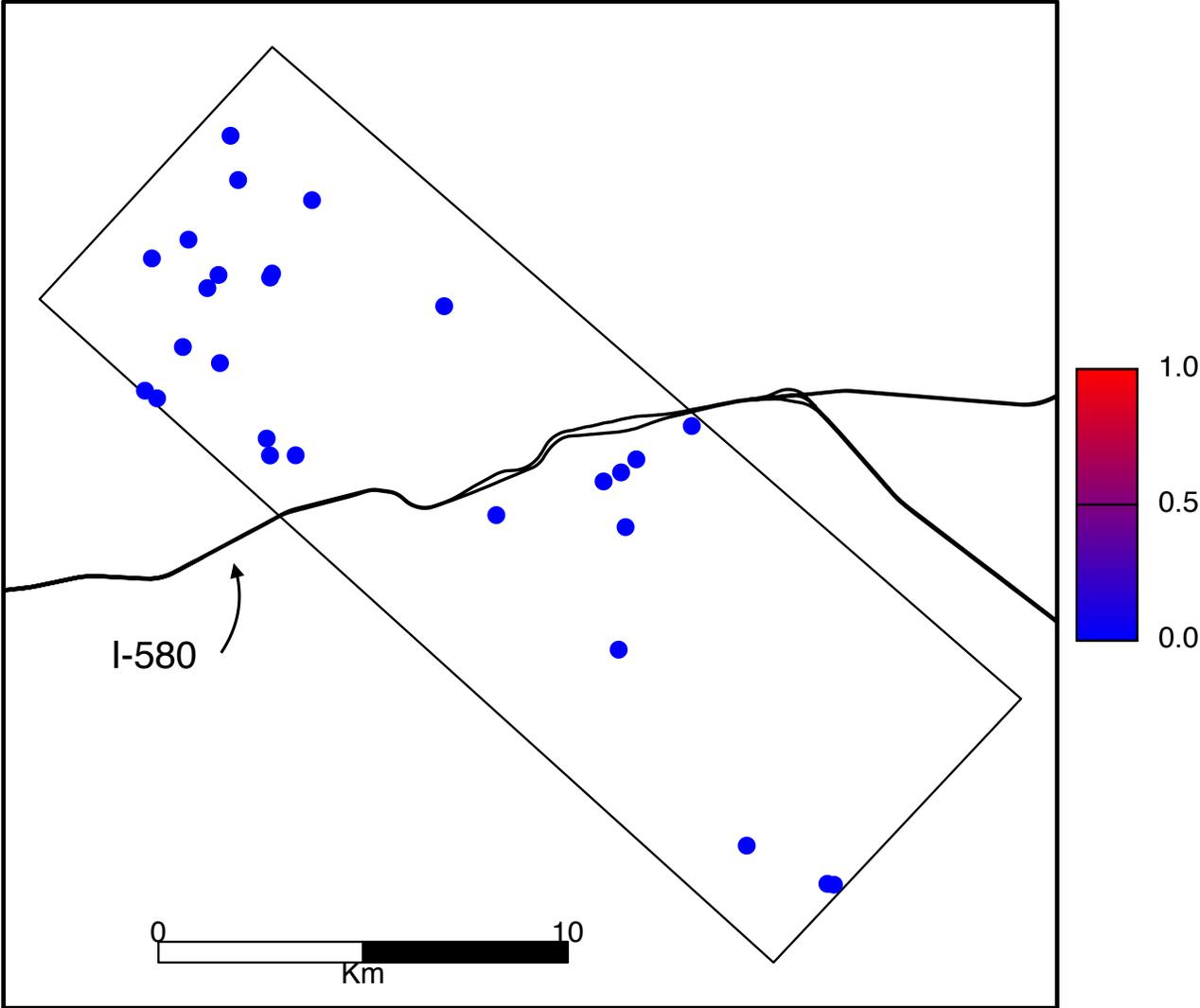


Figure S1: Region 1 ponds around I-580.

Region 2: CA-130

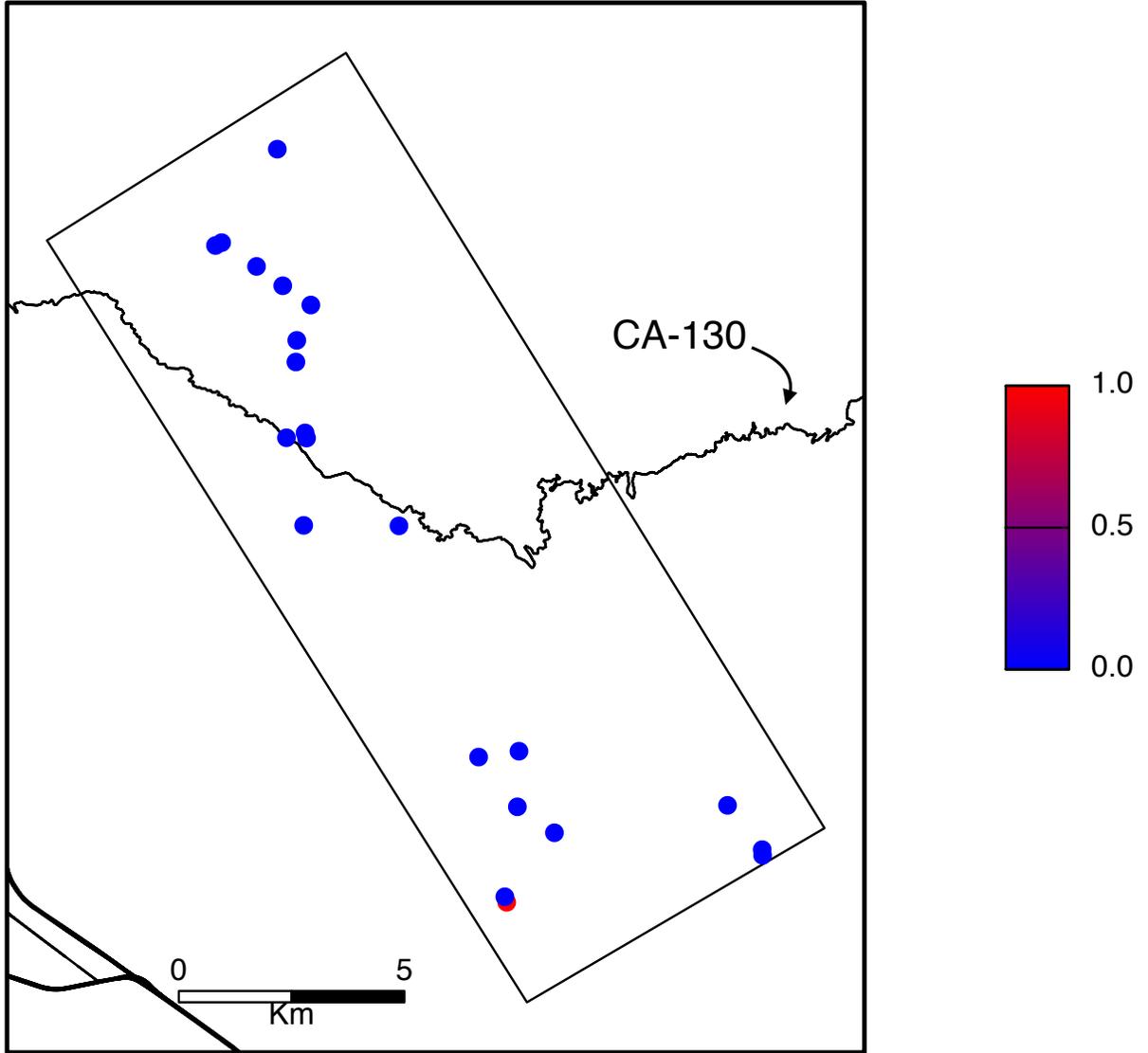


Figure S2: Region 2 ponds around CA-130.

Region 3: US-101 – San Juan Bautista

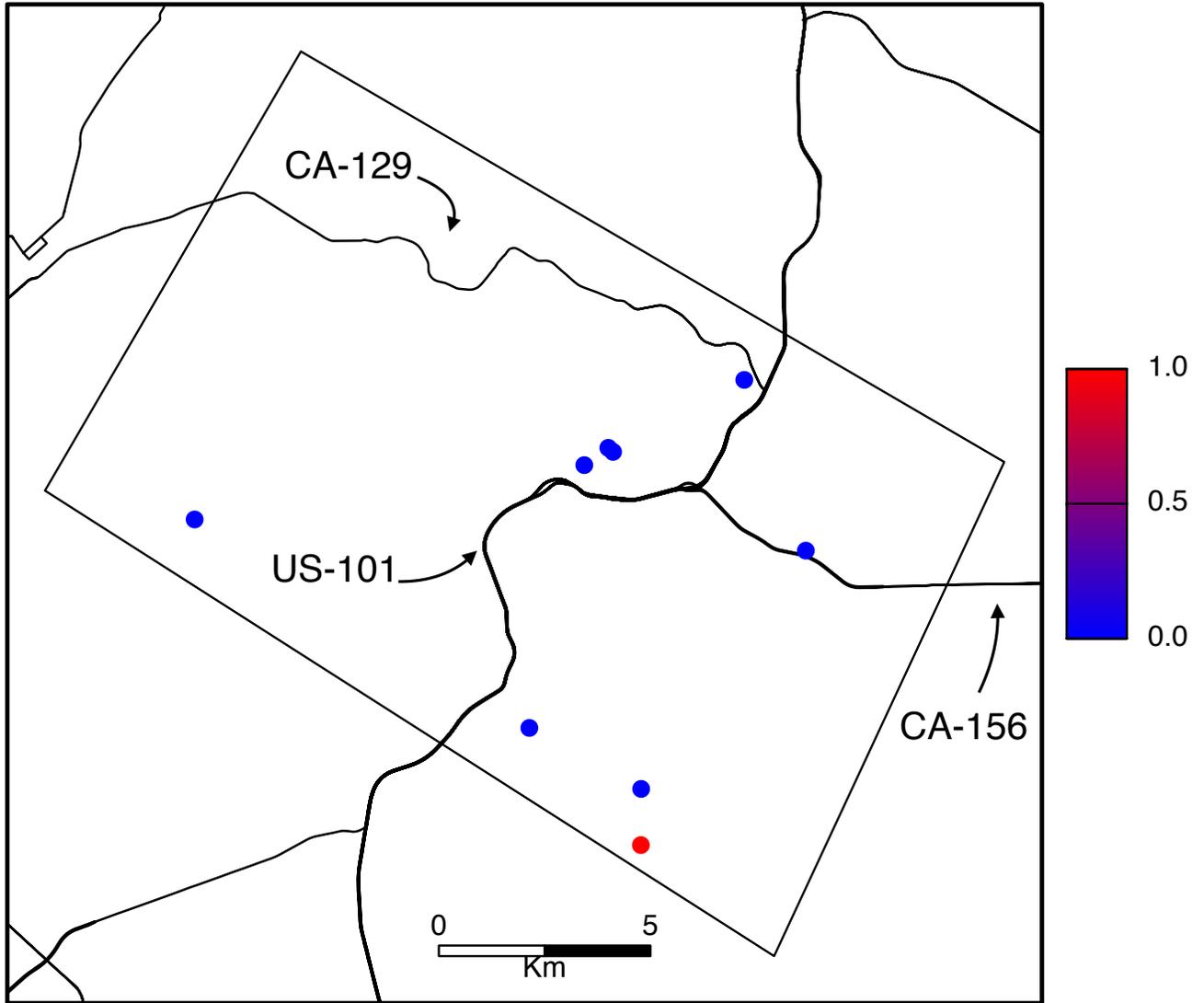


Figure S3: Region 3 ponds around US-101 in the vicinity of San Juan Bautista. Ponds were classified as on either the same or different sides of US-101 only.

Region 5: San Juan Canyon Road

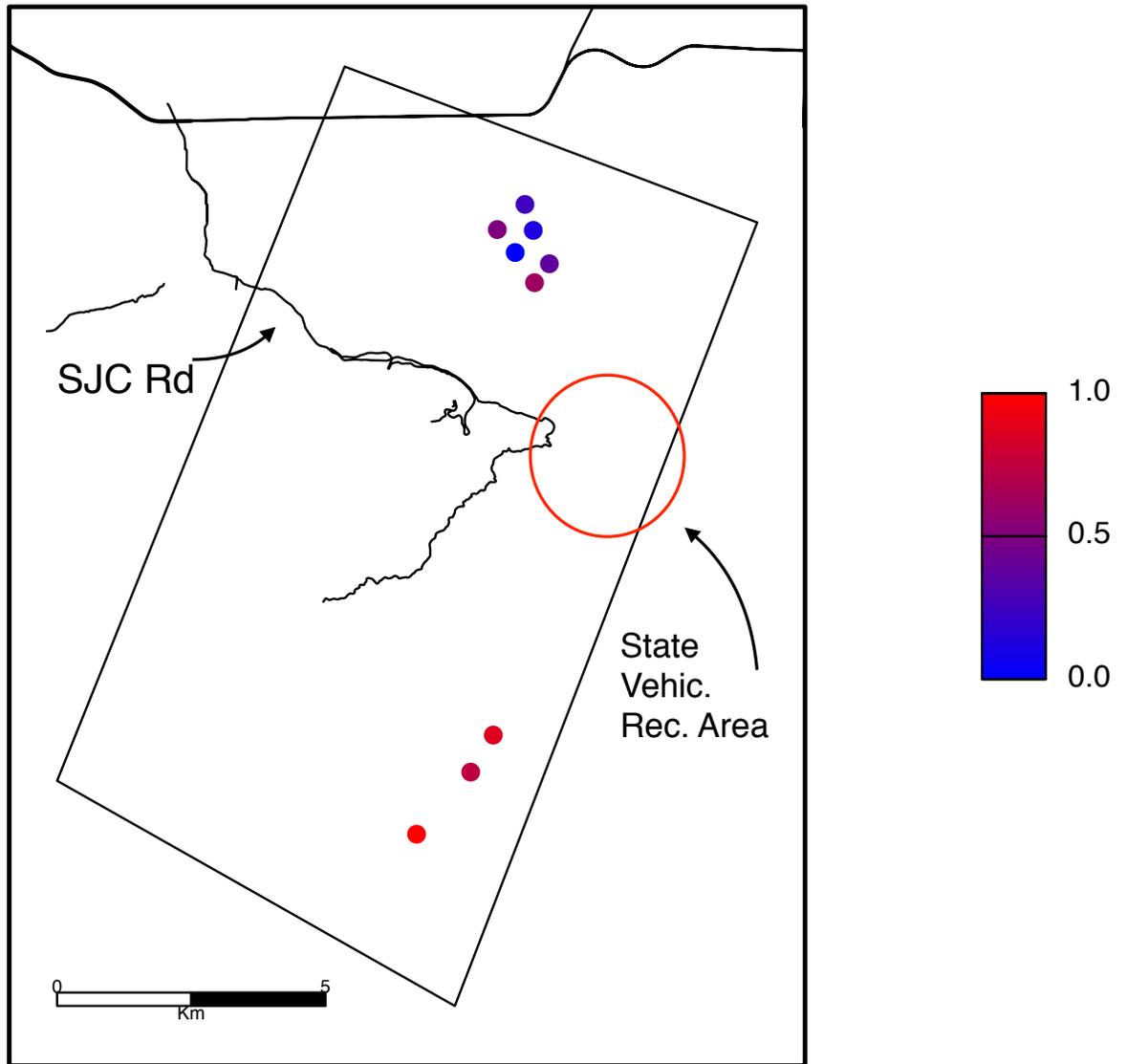


Figure S5: Region 5 ponds around San Juan Canyon Road (SJC Rd) and the Hollister Hills State Vehicular Recreation Area. The latter is comprised of unpaved, but densely spaced, roads. Ponds were classified as on the same or different sides of San Juan Canyon Road + the Recreation Area.

Region 6: CA-68

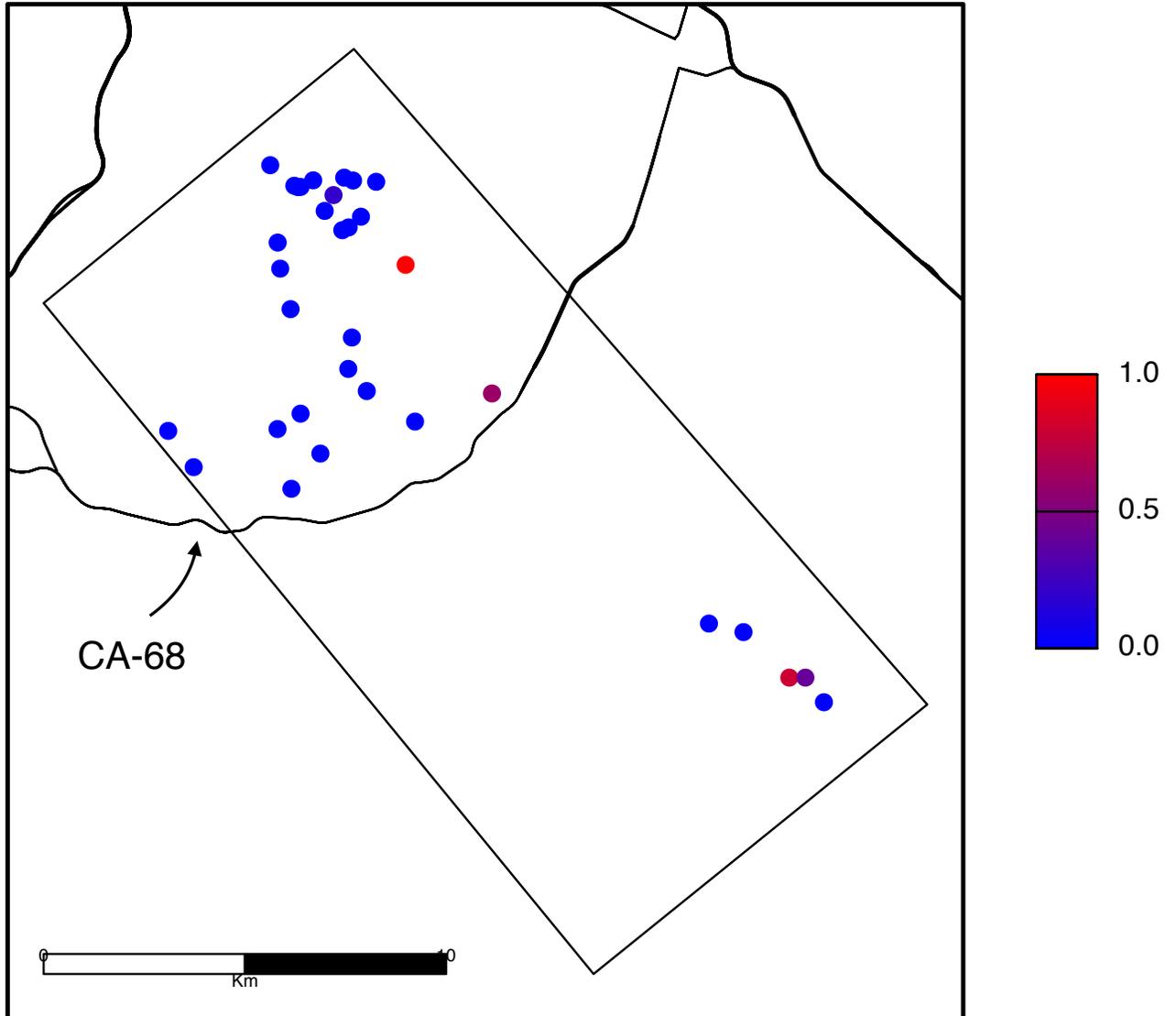


Figure S6: Region 6 ponds around CA-68.

Region 8: Salinas Valley Hybrid Region No Major Roads

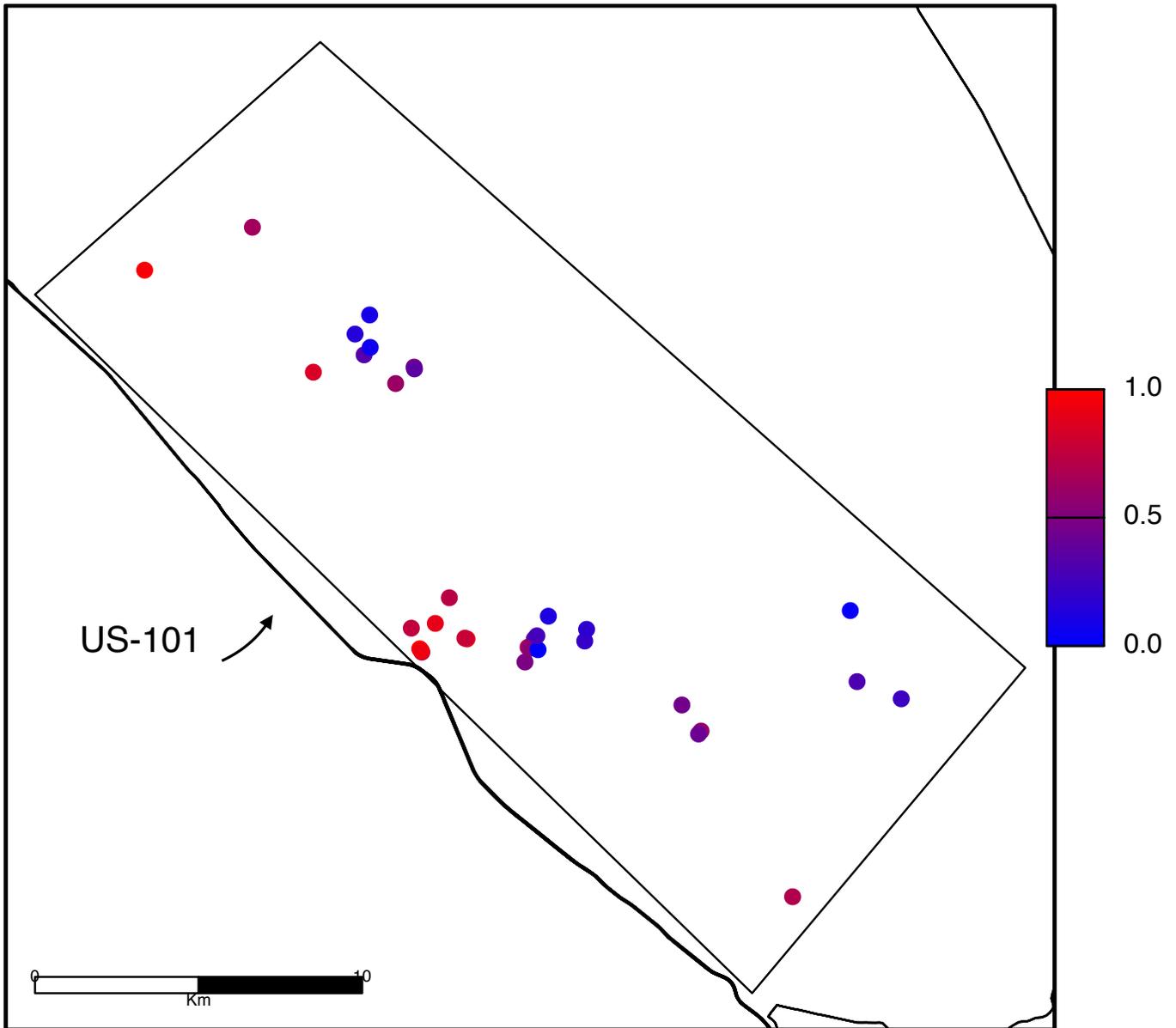


Figure S8: Region 8 ponds in the Salinas Valley north east of US-101. There are no intervening roads in this region, so all populations pairs are considered to be on the same side of the road.

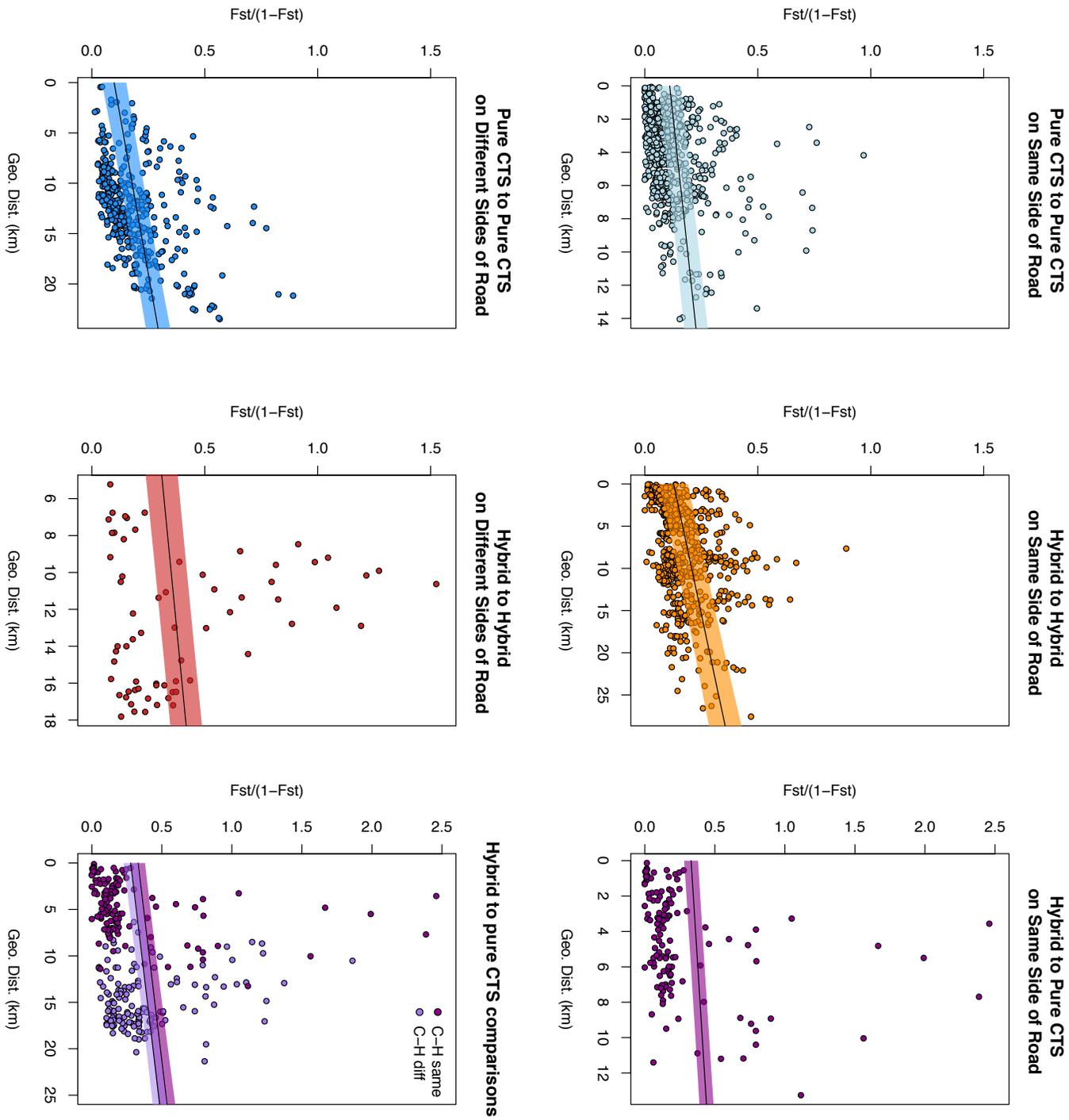


Figure S9: Visualizations of the relationship of F_{ST} to distance for each genotypic comparison-by-roadside group.

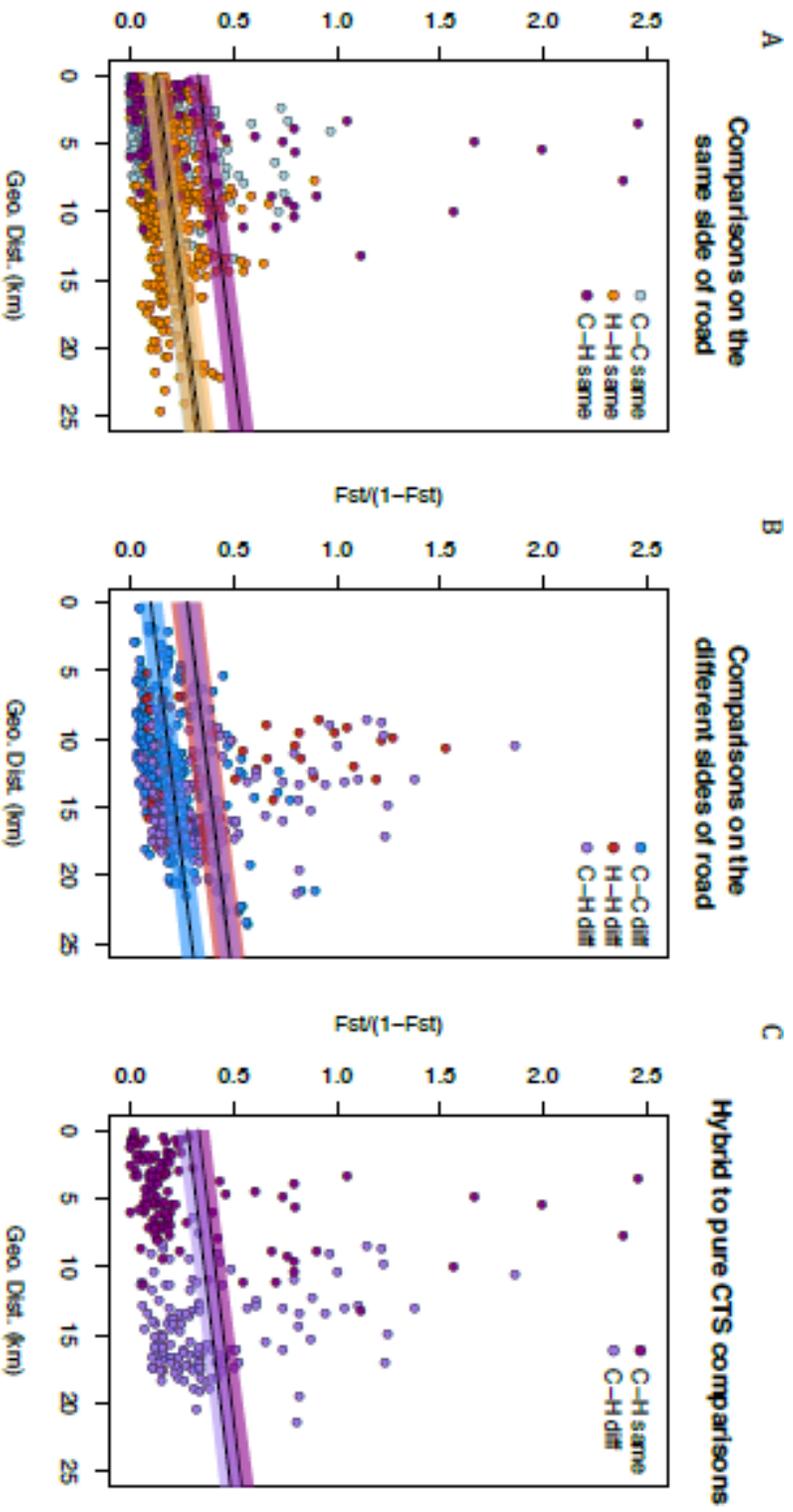


Figure S10: Contrasts between CTS-hybrid, CTS-CTS and hybrid-hybrid comparisons on the same side of the road (A) and different sides of roads (B). Contrast between CTS-hybrid comparisons on the same versus different sides of road (C).