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Character Displacement in the Midst of Substantial Background Evolution in Anolis Lizard Island Populations

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Title: Character displacement in the midst of background evolution in island populations of *Anolis* lizards: a spatiotemporal perspective

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Data Accessibility: Data and R code for reproducing the results presented here are available at datadryad.org (10.5061/dryad.pzgmsbcj0).

Abstract

Negative interactions between species can generate divergent selection that causes character displacement. However, other processes cause similar divergence. We use spatial and temporal replication of island populations of *Anolis* lizards to assess the importance of negative interactions in driving trait shifts. Previous work showed that the establishment of *Anolis sagrei* on islands drove resident *Anolis carolinensis* to perch higher and evolve larger toepads. To further test the interaction's causality and predictability, we resurveyed a subset of islands nine years later. *Anolis sagrei* had established on one island between surveys. We found that *A. carolinensis* on this island now perch higher and have larger toepads. However, toepad morphology change on this island was not distinct from shifts on six other islands whose *Anolis* community composition had not changed. Thus, the presence of *A. sagrei* only partly explains *A. carolinensis* trait variation across space and time. We also found that *A. carolinensis* on islands with previously established *A. sagrei* now perch higher than a decade ago, and that current *A. carolinensis* perch height is correlated with *A. sagrei* density. Our results suggest that character displacement likely interacts with other evolutionary processes in this system, and that temporal data are key to detecting such interactions.

Keywords: character displacement, interference competition, interspecific interaction, species invasion, parallel evolution, predictability

Introduction

Understanding the frequency with which interspecific interactions lead to repeatable patterns of diversification helps us understand the processes shaping variation in nature. Character displacement theory, for example, suggests that negative interactions between ecologically similar species generate divergent natural selection that can drive evolutionary change (reviewed in Pfennig and Pfennig 2012). Such divergence is thought to play a major role in niche partitioning, coexistence, community assembly, and adaptive radiation (reviewed in Schluter 2000, Pfennig and Pfennig 2012).

However, character displacement theory is difficult to test in the field (Stuart and Losos 2013, Germain et al. 2018). Models suggest that as negative interspecific interactions drive trait divergence, the intensity of said interactions will attenuate relatively rapidly, making them hard to detect (Connell 1980, Schluter 2000). As such, researchers typically use present patterns of divergence to infer how past interactions shaped past selection and past evolution. Such inference is often uncertain (Simberloff and Boecklen 1981, Stuart and Losos 2013) because similar divergence patterns can arise from many different processes (Stuart et al. 2017).

One approach to inferring process from pattern is to monitor novel interactions between ecologically similar species *through time* and test for whether and how character displacement unfolds. A model case comes from a colonization event that caused new interactions between two seed-eating Galápagos finch species (Grant and Grant 2006). In 1984, the large ground finch (*Geospiza magnirostris*) arrived on the island of Daphne Major, encountering a long-established population of the medium ground finch (*Geospiza fortis*). Following a severe drought and the consequent decline in seed abundance, the two species experienced increased competition for seeds and diverged in a key seed-use trait—beak size (Grant and Grant 2006).

The temporal span of that study—thirty years of pre- and post-interaction data for a single population of *G. fortis*—built a solid case for predictable, competition-driven character displacement on Daphne Major. Nevertheless, context mattered, as divergence in this system was contingent on more than just the presence of potential competitors. That is, observed patterns of stasis and change in beak size suggested that the strength of competition itself (and therefore the source of divergent selection) was controlled by the weather cycles that influence seed resources (Grant et al. 2017)—weather determined whether character displacement occurred (see Figure 2 in (Grant and Grant 2006)).

A second, more common way to make inferences about process from character displacement patterns is by comparing multiple populations within a focal taxon. Repeated character displacement *across space* can constitute strong evidence for competition-driven evolutionary divergence. For example, character displacement between sympatric interactors has been documented at multiple locations repeatedly and independently for several taxa, including stickleback (*e.g.*, Schluter and McPhail 1992), spadefoot toads (*e.g.*, Rice et al. 2009), and salamanders (*e.g.,* Adams 2010).

Spatial replication, as exemplified in comparative studies across populations, and temporal replication, as exemplified in long-term studies of single populations, can each help us understand the relative importance of character displacement amidst environmental contingencies and background evolution. However, these two approaches are more powerful when combined.

In a study taking advantage of the spatial replication offered by repeated species introductions to island populations, Stuart et al. (2014) examined habitat-use shifts and evolutionary change in one species of *Anolis* lizard following the arrival and establishment of a second species of anole. For millions of years, *Anolis carolinensis* was the likely only anole found in the southeastern United States as until recently, there were no other extant anoles. *Anolis sagrei* arrived to southern Florida in the late 1800s, was locally abundant by the late 1940s (Collette 1961), and has since spread through Florida and beyond (Campbell and Echternacht 2003). The two species are similar enough in habitat use and diet that they are expected to compete for resources (Collette 1961, Campbell 2000). In Mosquito Lagoon, Florida, Stuart et al. (2014) compared five, hectare-sized islands with only *A. carolinensis* to six similar islands where *A. sagrei* had established sympatric populations sometime between 1995 and 2010 (Campbell 2000). They found that on every island with *A. sagrei*, *A. carolinensis* perched higher (Figure S1). Moreover, on average, those *A. carolinensis* populations co-occurring with *A. sagrei* had larger toepads with a larger number of specialized, clingy scales called lamellae. A common garden study revealed that toepad variation had a heritable component, and a variety of other potential causes for this repeated evolution, including environmental differences and non-random migration, were ruled out (Stuart et al. 2014). Because arboreality across the ~400 species of anoles is associated with larger toepads that have more lamellae (Glossip and Losos 1997, Macrini et al. 2003), and because toepad area correlates positively with clinging ability (Elstrott and Irschick 2004), Stuart et al. (2014) concluded that *A. carolinensis* repeatedly adapted to a more arboreal lifestyle in no more than ~20 generations following the introduction of a congener.

Notably, however, the six islands with both *A. carolinensis* and *A. sagrei* (where *A. carolinensis* perched the highest) were not always the islands with the largest toepad area or lamella number (Stuart et al. 2014); see Figure S1 in this manuscript). That is, island by island, morphological character displacement in *A. carolinensis* was less consistent than the habitat shift induced by the presence of *A. sagrei*. This motivates further investigation of the magnitude and direction of morphological evolution that is facilitated by the behavioral response of *A. carolinensis* to *A. sagrei*.

In this paper, we add a temporal component to Stuart et al. (2014) by revisiting the islands of Mosquito Lagoon to test predictions about the repeatability of behavioral shifts and morphological character displacement across both space and time (Table 1). We report new data, collected in 2019, for *A. carolinensis* perch height and toepad morphology from six islands: three that had both *A. carolinensis* and *A. sagrei* in 2010 and still do (‘two-species’ islands); and, three that had only *A. carolinensis* in 2010 and 2019 (‘one-species’ islands). We compare these 2019 data to the data that were collected in 2010 (Stuart et al. 2014) to ask whether and how perch height and toepad traits changed through time on these islands (Question 1 in Table 1). These islands, whose species status did not change from 2010 to 2019 serve as a baseline and reveal whether character displacement is still evolving in this system, or whether *A. carolinensis* reached a stable adaptive optimum by 2010, in ~20 generations (Stuart et al. 2014).

We then report new data on perch height and toepad morphology from one island that had only *A. carolinensis* in 2010 but both species in 2019. We compare *A. carolinensis* perch height and toepad morphology in this single population before and after *A. sagrei* arrival (Question 2 in Table 1). We predicted that *A. carolinensis* on this island would perch higher and have larger toepads with more lamellae than they did prior to *A. sagrei* arrival. This comparison of change in toepad traits provides a complementary test of the conclusions previously drawn from comparative data (Stuart et al. 2014): that novel interactions with *A. sagrei* result in toepad divergence.

Finally, to better understand how variation in interspecific interactions might lead to observed variation in the magnitude of character displacement in this system (Figure S1; Stuart et al. 2014), we quantify finer-scale effects of *A. sagrei* presence on *A. carolinensis* perch height in two ways. First, we test whether average *A. carolinensis* perch heights correlate positively with *A. sagrei* population density (Question 3a). If yes, this suggests that variable character displacement across islands might depend in part on island-to-island variation in *A. sagrei* population dynamics. Second, *A. sagrei* density could affect *A. carolinensis* perch heights through different types of negative interactions, e.g., resource competition, interference competition, apparent competition, or intraguild predation (Schluter 2000). Testing among these is difficult. Some evidence suggests that interference competition is important in this *Anolis* system (Edwards and Lailvaux 2013, Culbertson and Herrmann 2019). In particular, *A. carolinensis* are substantially more likely than *A. sagrei* to flee from interspecific interactions, with males retreating upward more often than expected by chance (Culbertson and Herrmann 2019). Thus, we predicted that among *A. carolinensis* on two-species islands, individuals observed in the immediate vicinity of *A. sagrei* would perch higher than individuals observed with no *A. sagrei* nearby (Question 3b). This finding would further implicate agonistic, interference interactions in the perch height shift.

Materials and Methods

We studied seven islands in 2019 that were also studied in 2010 (Table 2). *Anolis carolinensis*, the focal species, was present on all seven islands in 2010 and 2019 (Stuart et al. 2014, this study). *Anolis sagrei* was present on three of the study islands in both 2010 and 2019 (Channel, North Twin, and Yang). *A. sagrei* was absent on three islands in both 2010 and 2019 (Hornet, Crescent, and Osprey). These six islands are referred to in this manuscript as 'control' islands, for brevity, because their species category (i.e., one- versus two-species) did not change from 2010 to 2019. One island, South Twin, was a one-species island in 2010 and a two-species island in 2019. *Anolis sagrei* arrived on South Twin sometime between 2010 and 2018, per island surveys by Y.E.S., T.S.C., and K.C.S. on July 7, 2018.

From May 29 to June 14, 2019, we visited each of the seven islands on multiple days. One to five workers landed on an island by about 7:00 am and stayed until about 12:30 pm. We walked through the habitat until an undisturbed *A. carolinensis* individual was observed. We noted the lizard's perch and attempted to capture it by hand or with lasso poles. We recorded perch height (using tape measures) and sex for each lizard. Sexes were distinguished based on body size and shape, including dewlap characteristics, as well as the presence of externally visible hemipenes and enlarged post-anal scales in males that were captured. We transported captured lizards to field housing for toepad data collection. After measuring toepads, we numbered each lizard with permanent ink to avoid repeated measures of the same individual and released it where captured, typically within 30 hours.

In field housing, we anesthetized lizards using isoflurane. While the lizard was anesthetized, we used digital calipers to measure snout-vent length (SVL) to the nearest mm as a measure of body size. We then used a digital scanner at 2400dpi to image the fourth (longest) toe of each hind foot, following (Glossip and Losos 1997, Stuart et al. 2014).

We used FIJI (Schindelin et al. 2012) and the plugin ObjectJ v1.04r (Vischer and Nastase 2018) to count lamellae and measure toepad area from the digital scans. Following Stuart et al. (2014), we counted the number of lamellae on the third and fourth (*i.e.*, distal-most) phalanges of each toe. We then traced the region encompassed by those lamellae to measure the area of the left and right toepad. Lamella counts and pad areas were averaged across sides. Y.E.S. conducted all phenotyping (as they did for the 2010 data), after practicing on a burn-in sample of 50 images. Information on source island was not attached to toepad photographs during phenotyping.

Histograms of lamella number and toepad area were normally distributed for both 2010 and 2019 data, though 2010 toepad area showed a heavy left tail. We ran linear regressions of toepad traits ~ SVL x sex with females as the baseline to assess the need for size-correction prior to further analysis. In the 2019 data, average toepad area was positively related to SVL and the relationship varied by sex ($\beta_{\text{size}} = 0.09$, $\beta_{\text{male}} = -2.70$, $\beta_{\text{interaction}} = 0.06$, all $p < 0.0001$, Adj. $R^2_{\text{model}} = 0.88$). Average lamella number did not vary with SVL ($\beta_{\text{size}} = 0.05$, $\beta_{\text{male}} = 1.48$, $\beta_{\text{interaction}} = -0.01$, all $p > 0.19$, Adj. $R^2_{\text{model}} = 0.27$). In the 2010 data also, average toepad area was positively related to SVL and the relationship differed by sex ($\beta_{\text{size}} = 0.08$, $\beta_{\text{male}} = -2.76$, $\beta_{\text{interaction}} = 0.06$, all $p < 0.0003$, Adj. $R^2_{\text{model}} = 0.87$). Again, lamella number did not vary with SVL ($\beta_{\text{size}} = 0.06$, $\beta_{\text{male}} = 1.61$, $\beta_{\text{interaction}} = -0.01$, all $p > 0.18$, Adj. $R^2_{\text{model}} = 0.27$). Thus, we corrected toepad area but not lamella number for SVL.

We size-corrected toepad area separately for males and females, due to the significant interaction between sex and SVL in the regression against toepad area. Size correction used: $M_{s,i} = M_{0,i} * (L_s/L_{0,i})^b$, where $M_{s,i}$ is the size-corrected toepad area for individual i, $M_{0,i}$ is the non-size-corrected toepad area for individual i, L_s is the overall mean for SVL across all individuals of the given sex, and $L_{0,i}$ is the SVL of individual *i*. *b* is the common within-group slope calculated from a linear mixed model of the log of the individual toepad area ($\log_{10}(M_{0,i})$) regressed on the log of the individual SVL ($\log_{10}(L_{0,i})$) (Lleonart et al. 2000, Oke et al. 2016). In this regression, we included year as a fixed effect and island as a random effect. This mixed model was run using the *lmer* function (in the *lme4* package in R; Bates et al. 2014), coded as follows: toepad area ~ SVL + year + (1|island). *b* for females and males was 1.37 (s.e. = 0.12) and 1.97 (s.e. = 0.06), respectively.

Data Analysis

To investigate whether *A. carolinensis* on the six control islands (three one-species and three two-species islands) changed their perch heights, toepad area, and lamella number from 2010 to 2019 (Question 1), we built a linear mixed effects model and combined data from 2010 (Stuart et al. 2014) and 2019 (this study). Our statistical null hypothesis was that we would find no change in *A. carolinensis* perch height or morphology because there had been no changes in the presence of *A. sagrei.* This effectively posits that *A. carolinensis* trait shifts in response to *A. sagrei* had stabilized by 2010. Perch height, size-corrected toepad area, and lamella number were response variables, with each modeled separately. Fixed effects were year, species category (one- or two-species), a year x species category interaction, and sex. Island was included as a random effect. We used *lme* in the R package *nlme* (Pinheiro et al. 2014) coding models as follows: response ~ year x category + sex, random = ~1|island. Here, the year effect evaluates Question 1 by revealing whether these populations changed from 2010 to 2019; the year x species category effect tests whether changes with time differed between one- and two-species islands.

To compare habitat use and morphology by *A. carolinensis* on South Twin in 2019 versus 2010 (Question 2), we regressed perch height, size-corrected toepad area, and lamella number (with each modeled separately) against year and sex. We used *lm* in R's *stats* package, coded as follows: response ~ year + sex. A positive, significant effect of year suggests an increase in 2019 relative to 2010.

We further tested Question 2 using models that pooled all islands. These models tested whether change observed on South Twin was significantly different from change observed on the six control islands. Again using *lm*, we regressed perch height, size-corrected toepad area, and lamella number, in turn, against year, island, a year x island interaction, and sex. We built the model so that South Twin females from 2010 would be the baseline comparison group. In this way, a significant year effect suggests that all else equal, lizards on South Twin have a different trait mean in 2019 than in 2010. A significant island effect means that a given island has a different base trait value than South Twin. A significant year x island effect means that any change from 2010 to 2019 on that island differed from temporal change observed on South Twin.

We next asked whether *A. carolinensis* perch height depends on the population density of *A. sagrei* (Question 3a). In 2019, we estimated the relative density of *A. sagrei* across islands using observation-per-unit-time surveys. One researcher (G.C.) walked each two-species island twice, including South Twin, conducting an hour-long survey for *A. sagrei* between 7:15 and 9:00 am. The average of these two survey counts is a proxy for island-wide density. We regressed, using *lm*, island mean perch height against island mean *A. sagrei* count.

Previous studies showed that the presence of *A. sagrei* causes an increase in *A. carolinensis* perch height (Campbell 2000, Stuart et al. 2014, Pringle et al. 2019). If this is partly due to an agonistic interaction (Edwards and Lailvaux 2013, Culbertson and Herrmann 2019), we expect that individual *A. carolinenis* on two-species islands should perch higher when *A. sagrei* is nearby (Question 3b). In both 2010 (Stuart et al. 2014) and 2019, for every observed *A. carolinensis* perch height, we noted whether any *A. sagrei* individuals were within a ~1m-radius cylinder, centered at the observed perch site and stretching from ground to sky. We used a linear mixed model to regress *A. carolinensis* perch height against the presence or absence of *A. sagrei* near the perch with *A. carolinensis* sex as a fixed effect. Island was included as a random effect. As above, we used *lme*, coding the model as follows: perch height ~ sagrei presence + sex, random = ~1|island. We ran separate regressions for 2010 and 2019 data because South Twin was a one-species island in 2010 and so replicate numbers differ from year to year.

Results

Question 1

Perch height: For control islands, *i.e.*, islands whose species category (one-species or two-species) did not change between 2010 and 2019, a linear mixed model detected a significant interaction between year and species category. Compared to one-species islands, two-species islands with *A. sagrei* showed significant increases in *A. carolinensis* perch heights from 2010 to 2019 (Table 3, Figure 1).

Toepad area: For control islands, a linear mixed model found no year effect and no year by species category interaction for toepad area (Table 4; Figure 2). The difference in toepad area between islands with and without *A. sagrei* was in the same direction as observed in Stuart et al. (2014) (compare Figure 2 to Figure S1), though the difference detected here using a smaller sample of islands was not significant ($p = 0.121$).

Lamella number: For control islands, lamella number did not change between 2010 and 2019, and we observed no year by species category interaction (Table 5, Figure 2). The difference in lamella number between islands with and without *A. sagrei* was in the same direction as observed in Stuart et al. (2014), though the difference detected here using a smaller sample of islands was not significant ($p = 0.154$).

Question 2

Linear models for South Twin only

(i) Perch Height: A linear model with just lizards from South Twin showed that *Anolis carolinensis* on South Twin perched 54cm higher in 2019 compared to 2010 ($p = 0.0002$; $mean^{+1se}_{-1se}$; $119.7^{128.4}_{111.0}$ cm to $173.4^{186.0}_{160.8}$ cm). The same model showed that males perched higher than females by 49cm, across years, all else equal ($p = 0.0011$).

(ii) Toepad Area: As predicted, size-corrected toepad area for *A. carolinensis* increased on South Twin from 2010 to 2019 ($mean^{+1se}_{-1se}$; $3.52^{3.63}_{3.40}$ mm^2 to $3.76^{3.86}_{3.65}$ mm^2), an increase of approximately 7%. Linear regression with year and sex as predictors, and 2010 females as the baseline, revealed a positive, significant effect of 2019 versus 2010 ($\beta_{2019} = 0.12$, $p = 0.028$). Males had toepads with significantly larger areas ($\beta_{male} = 1.55$, $p < 0.001$).

(iii) Lamella number: Although mean lamella number for *A. carolinensis* was higher on South Twin in 2019 relative to 2010 ($mean^{+1se}_{-1se}$; $23.28^{23.45}_{23.11}$ lamellae to $23.77^{23.96}_{23.59}$ lamellae), an increase of approximately 2%, though the increase was not significantly different from zero ($\beta_{2019} = 0.37$, $p = 0.079$) Males had toepads with significantly more lamellae than females ($\beta_{male} = 1.52$, $p < 0.001$).

Is change on South Twin between 2010 and 2019 significantly different from change on control islands?

(i) Perch Height: We used a linear model with all islands to ask whether perch height change on South Twin was different from changes on control islands whose species number did not change between 2010 and 2019. We structured the model so that South Twin served as the baseline against which other islands were compared. The year effect was significant ($p < 0.001$), again showing that *A. carolinensis* perch height increased on South Twin all else equal (by 53.9cm, Table 6). Year x island interactions revealed significantly different perch height shifts by *A. carolinensis* on four other islands compared to South Twin (Table 6). Overall, *A. carolinensis* on one-species islands showed either an increase (Hornet) or a decrease (Osprey and Crescent) in perch height from 2010 to 2019, relative to South Twin. On two-species islands, *A carolinensis* on Yang Island increased more than South Twin. The year x island interaction was not significant for Channel or North Twin, suggesting that these islands increased their perch height similarly to South Twin (Table 6). Summing the coefficient of the year effect with the island specific coefficient for the year x island interaction gives the model's best estimates for 2010 to 2019 change on each island (final column, Table 6). The rank order of perch height change from positive to *negative* was: Yang, Channel, South Twin, Hornet, North Twin, *Osprey*, and *Crescent* (Table 6).

(ii) Toepad Area: For size-corrected toepad area, the effect of year was positive and significant ($p = 0.034$; Table 7), again showing that *A. carolinensis* toepad area increased on South Twin from 2010 to 2019. The year x island effects were predominantly negative, though none were significant, meaning the toepad area increase on South Twin was not statistically distinguishable from changes on any other island. The rank order of toepad area change from positive to *negative* was: Yang, South Twin, Crescent, Channel, Hornet, North Twin, and *Osprey* (Table 7).

(iii) Lamella Number: For lamella number, the effect of year was positive and significant ($p = 0.046$; Table 8), showing that South Twin *A. carolinensis* had more lamellae in 2019 than in 2010. The year x island effects were predominantly negative, but significant on only one island (Yang; Table 8). Thus, our results suggest that the lamella count increase on South Twin was not statistically distinguishable from changes on other islands. The rank order of lamella number change from positive to *negative* was: Hornet, South Twin, Channel, *Crescent, Osprey*, *North Twin*, and *Yang* (Table 8).

Question 3a.

Does variation in the island-wide density of A. sagrei *explain population-level variation in* A. carolinensis *perch height across islands?*

Average counts during hour-long surveys for *A. sagrei* in 2019 were 18.5 (South Twin), 32.5 (North Twin), 41.5 (Channel), and 43.0 (Yang). Mean *A. carolinensis* perch height was positively related to average *A. sagrei* count (Figure 3). For every additional *A. sagrei*, the mean perch height of *A. carolinensis* increased by 3.4cm ($p = 0.0002$). A regression including only the two-species islands from 2019 showed a similar effect with only four island means (3.8cm perch increase per additional *A. sagrei*) but was only significant at the 0.10 level ($p = 0.09$).

Question 3b.

Does variation in the local presence of A. sagrei *explain individual-level variation in* A. carolinensis *perch height within islands?*

In 2019, the presence of *A. sagrei* near an *A. carolinensis* perch was significantly correlated with a 55cm increase in *A. carolinensis* perch height ($p < 0.0001$; Table 9A; Figure 4A). Sex differences in perch height disappeared in this model ($p = 0.51$), suggesting that male and female *A. carolinensis* are perching similarly once nearby *A. sagrei* are accounted for. The opposite result was found in 2010. *A. sagrei* nearby was negatively correlated with *A. carolinensis* perch height on two species islands ($\beta_{nearby} = -43$cm, $p = 0.01$; Table 9B), whereas being male was positively correlated ($\beta_{male} = 50$cm, $p = 0.004$; Table 9; Figure 4B).

Discussion

Species introductions can establish replicated competitive arenas that reveal the frequency and repeatability of character displacement (Stuart and Losos 2013). A drawback, however, to such natural experiments is that pre-introduction information is often lacking. Instead, allopatric populations are used as proxies for an ancestral state and so the reliability of inference depends on the reliability of the proxy. Long term datasets can provide pre-introduction data and thereby improve inference because a given population serves as its own baseline (e.g., Grant and Grant 2006). In this study, we added a temporal component to a study of character displacement that previously documented divergence spatially, across island replicates at a single point in time.

Sampling nine years after that study reveals that the coarse pattern of repeated character displacement observed in this system remains. This is particularly true for the behavioral perch height response of *A. carolinensis* to *A. sagrei*, which remained large and largely predictable at both the across-island and within-island levels. However, morphological evolution of toepads is not linked as strongly as predicted to the observed behavioral change, indicating that the process of character displacement is nuanced, dynamic, and requires investigation across both space and time to contextualize it amidst background evolution.

Perch height change

In agreement with prior results (Stuart et al. 2014), we found that *A. sagrei* presence is a significant predictor of *A. carolinensis* perch height. On average, in 2019, *A. carolinensis* perched higher when sympatric with *A. sagrei*. This was true on islands whose species category (one versus two species) did not change between 2010 and 2019 (addressing Question 1), and on South Twin, which was a two-species island in 2019 but not 2010 (addressing Question 2). Together, these results corroborate experimental and comparative evidence that *A. sagrei* presence is the cause of habitat-use shifts in *A. carolinensis* (Stuart et al. 2014, Pringle et al. 2019). Behavioral plasticity is most likely responsible for initial shifts in perch height. Individual *A. carolinensis* alter their habitat use in real-time when an *A. sagrei* individual is introduced nearby (Culbertson and Herrmann 2019), and introduction experiments showed detectable, population-level shifts within weeks (Campbell 2000, Stuart et al. 2014). However, we cannot rule out an evolved component to this behavioral shift, especially as it persists across generations.

Our temporal approach further reveals that shifts in *A. carolinensis* perch height are not one-time events that stabilize shortly after *A. sagrei* arrival. We found that *A. carolinensis* on two-species islands perched higher in 2019 than they did in sympatry in 2010. That this increase did not occur on one-species islands again implicates *A. sagrei* as the cause and suggests that the intensity of the interspecific interaction has magnified over time.

Change in morphology

The establishment of *A. sagrei* on South Twin after the resident *A. carolinensis* population had been studied in 2010 allowed us to test the extent to which morphological character displacement in this system is predictable (Question 2). Comparing *A. carolinensis* on South Twin in 2010 and 2019, we found that toepad area was significantly higher in 2019 than in 2010, and there was a trend toward more lamellae in 2019 (p = 0.079), despite sampling fewer islands than Stuart et al. (2014). Had we restricted our 2019 sampling to this single island, we would have concluded that toepad evolution is predictable in this system. There are three ways, however, in which additional results from this study complicate our prior understanding of morphological character displacement in this system (Stuart et al. 2014).

First, the toepad change observed on South Twin from 2010 to 2019 was statistically indistinguishable from the observed change on both one- and two-species islands, where zero change was expected (Table 7, Table 8). Thus, despite some evidence for adaptive morphological evolution arising due to a novel negative interaction, that signal does not stand out against background evolution in control populations.

Second, we detected no significant differences in toepad morphology between *A. carolinensis* on two-species and one-species control islands (Question 1, Table 4 and 5). That is, the morphological pattern observed across island replicates in 2010 (Stuart et al. 2014) disappeared. This is explainable at least in part by the fact that we sampled only six islands in 2019, whereas Stuart et al. (2014) sampled 11 islands in 2010. Subsampling all possible combinations of three one-species islands and three two-species islands from data collected in 2010 (Supporting Information), we found that the effect sizes of the differences in toepad morphology between one- and two-species islands are comparable in 2019 relative to 2010, suggesting low statistical power as an explanation (Figure S2, Figure S3, Figure S4). Nevertheless, the fact that it matters which and how many islands were sampled underscores island-to-island variability in the toepad response to perch height use (Figure S1).

Third, despite a large increase in perch height on the two-species control islands from 2010 to 2019 (Table 3), we did not see an increase in toepad size or lamellae number through time on two-species islands relative to one species islands (Tables 4 and 5). This finding that dynamic and highly consistent behavioral interactions do not lead to equally striking morphological character displacement suggests two possibilities. One, toepad evolution lags behind behavioral change. Though toepad evolution is inferred to be fast in this system (Stuart et al. 2014), perhaps Channel and Yang, for example, only recently experienced an increase in *A. sagrei* density (see discussion below), and *A. carolinensis* are therefore still evolving toward new optima. Two, perhaps the long-standing adaptive assumption of a finely-tuned and selection-driven correlation between perch height and toepad morphology in this system is wrong. Although this correlation holds across *Anolis* species (Glossip and Losos 1997, Macrini et al. 2003), past research on these relationships within species of *Anolis* has been equivocal and remains in need of both conceptual and empirical synthesis (Irschick and Losos 1998, Irschick and Losos 1999, Sathe and Husak 2015, Kamath and Losos 2017). Specific questions that remain unanswered include: How much of a fitness advantage do *A. carolinensis* gain from having larger toepads or more lamellae, what is the shape of the fitness function, and to what extent does an advantage depend on the frequency and nature of their encounters with each other, with *A. sagrei,* and with other members of their ecological community (*e.g.,* predators)? Answering these questions will assess the utility of character displacement *as a process* in explaining both the rapid morphological evolution and repeated adaptive radiation for which *Anolis* lizards are well-known.

In general, there is growing evidence of fuzzy relationships between behavioral responses and other phenotypic traits that have long been thought of as tightly linked under character displacement. Indeed, Germain et al. (2018) have argued that an approach to character displacement that focuses on specific traits and trait correlations is both limited and biased, and instead suggest focusing on quantifying the competition that is central to character displacement using invasibility experiments. Nevertheless, they acknowledge that such experiments are not always feasible or ethical, particularly for animal taxa. Thus, more nuanced observational research on the day-to-day and generation-to-generation processes of character displacement remains necessary, and we posit that expansive spatiotemporal sampling efforts such as ours represent an important step.

Changing species interactions and habitat use

One avenue for building a nuanced understanding of character displacement in this system is to focus on how interactions between individual *A. carolinensis* and *A. sagrei* change through time. For example, interspecific individual interactions likely become more frequent with increasing population density. Across islands in 2019, *A. carolinensis* perch height was positively related to *Anolis sagrei* density (Figure 3; consistent with Prediction 3a). And, we found that, on two-species islands, a greater proportion of individual *A. carolinensis* were located nearby to an *A. sagrei* individual in 2019 compared to 2010 (61% vs. 24%; Supporting Information), consistent with an increase in *A. sagrei* density from 2010 to 2019 (though see below for methodological considerations). Thus, we tentatively attribute the continuing upward shift of *A. carolinensis* on two-species control islands to increases in *A. sagrei* density. We speculate that the initial perch height increase in *A. carolinensis* incited by *A. sagrei* arrival is a first step in a series of potential subsequent perch height shifts, which are modulated by changes in *A. sagrei* population density over multiple generations.

Although a correlation between *A. carolinensis* perch height and *A. sagrei* density explains variation in population-level behavior across islands, this correlation alone does not pinpoint a specific mechanism underlying negative interspecific interactions. Support for our hypothesis that direct, agonistic interactions contribute to shifts in *A. carolinensis* perch height appeared equivocal; in 2019 *A. carolinensis* perched 52cm higher when *A. sagrei* individuals were spotted within a 1m-radius of the focal lizard (consistent with Prediction 3b), but in 2010 the correlation between local *A. sagrei* presence and *A. carolinensis* perch height was negative (in contradiction with Prediction 3b) (Figure 4). Although direct interference does occur between these species and appears to be asymmetric in favor of *A. sagrei* (Edwards and Lailvaux 2013, Culbertson and Herrmann 2019), there is also evidence for both resource competition and intraguild predation in this system (Campbell 2000, Gerber and Echternacht 2000). In particular, *A. sagrei* do prey on *A. carolinensis* hatchlings (Campbell 2000), which could select for individuals that either learn to perch higher or that do so instinctively. The relative importance of these interactions in driving habitat shifts and subsequent character displacement warrants continued exploration (Grether et al. 2017, Stuart et al. 2017).

Why did we see evidence for interference competition in 2019 but not 2010 (Figure 4)? Several methodological and biological explanations are possible. First, we note that our methods for locating nearby *A. sagrei* upon observing an *A. carolinensis* were implemented far more rigorously in 2019 than in 2010. A more rigorous search for *A. sagrei* upon observing *A. carolinensis* could also partly explain the large increase from 2010 to 2019 in the number of observations of *A. carolinensis* where *A. sagrei* was detected nearby (from 24% to 61%). Our efforts were more focused in 2019 in part because intervening research (Culbertson and Herrmann 2019, N.C.H & A.K., *unpubl.*) suggested that the importance of interference competition in this system has been overlooked. We acknowledge that it is convenient that our more rigorous effort to search for *A. sagrei* coincided with obtaining the results we predicted and that it is possible that our more targeted focus on interference competition unconsciously biased our data collection in 2019. More generally, this reveals a challenge of long-term field research—how can we update and refine our hypotheses to take full advantage of repeated data collection while maintaining methodological consistency and guarding against confirmation bias?

Potential biological explanations also exist for opposite effects of nearby *A. sagrei* presence on individual *A. carolinensis* perch height in 2010 and 2019. These biological explanations illuminate some compelling directions for furthering our understanding of character displacement in this system. First, sex seems to be interacting with local *A. sagrei* presence to predict *A. carolinensis* perch height differences across years. That is, in 2010, nearby *A. sagrei* presence did not influence *A. carolinensis* perch heights but sex did. In 2019, sex had no effect, but nearby *A. sagrei* did. Male and female anoles differ in myriad aspects of their ecology (Lister and Aguayo 1992, Nunez et al. 1997, Butler et al. 2000), and are likely to interact differently with a congeneric competitor. Dissecting these differences might provide another way to understand the processes underlying non-repeated variation in perch height shifts and morphological character displacement. Second, density-dependent changes in *A. sagrei* behavior (*e.g.,* increased aggression at higher densities) might have altered the nature of interactions between the two congeners, precipitating an emergent shift in how *A. carolinensis* respond to *A. sagrei* (Peiman and Robinson 2010).

Conclusion

This study was motivated by the observation that *A. sagrei* arrived on South Twin Island after baseline data were collected on resident *A. carolinensis* in 2010. Would character displacement by *A. carolinensis* proceed on this island as it had between 1995 and 2010 on other islands in Mosquito Lagoon (Stuart et al. 2014)? Yes, but with an important qualification. *A. carolinensis* on South Twin now perch higher and have larger toepads, but these changes on South Twin are on par with background changes by *A. carolinensis* between 2010 and 2019 on islands whose *Anolis* community did not change.

Using replicated sampling across both space and time, we were able to move beyond simply asking whether character displacement occurs and could instead dissect the pattern more finely to reveal underlying processes. We see consistent evidence that co-occurring with a congener drives behavioral shifts across multiple spatial, temporal, and organizational scales, but that subsequent morphological shifts were not as consistent as we expected. Our results make it clear that we need to employ complementary approaches incorporating behavioral, ecological, biomechanical, and quantitative genetic data to better understand the processes by which patterns of character displacement arise and are maintained. Investigating density dependence, sex-based ecological differences, and the underlying functional bases of character displacement hold particular promise.

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Tables

Table 1. Study questions.

Question 1	Has *A. carolinensis* perch height and toepad morphology shifted on islands whose anole communities did not change between 2010 and 2019?
Question 2	Has *A. carolinensis* perch height and toepad morphology shifted on one island with a recent *A. sagrei* arrival? *Prediction*: On the island with recent *A. sagrei* arrival, *A. carolinensis* would perch higher and have larger toepads with more lamellae than they did prior to *A. sagrei* arrival.
Question 3a	Does variation in the island-wide density of *A. sagrei* explain population-level variation in *A. carolinensis* perch height across islands? *Prediction*: Islands with higher density of *A. sagrei* would have higher mean perch heights for *A. carolinensis.*
Question 3b	Does variation in the local presence of *A. sagrei* explain variation among individuals in *A. carolinensis* perch height within islands? *Prediction*: Among *Anolis carolinensis* on two-species islands, individuals would perch higher when individual *A. sagrei* were observed nearby.

Table 2. *Anolis sagrei* presence status, *A. carolinensis* perch height sample size, and *A. carolinensis* morphology sample size by island for 2010 and 2019. For sample sizes, males (m) are listed before the "/" and females (f) after. South Twin, which was a one-species island in 2010 and a two-species island in 2019, is indicated in bold.

		Two-species?		Perch height sample size (m/f)		Morphology sample size (m/f)	
Island	Code	2010	2019	2010	2019	2010	2019
North Twin	ntw	Yes	Yes	48 / 21	45 / 26	33 / 11	30 / 18
Channel	cha	Yes	Yes	51 / 15	45 / 30	38 / 15	31 / 16
Yang	yan	Yes	Yes	57 / 14	35 / 16	41 / 16	31 / 12
South Twin	**stw**	**No†**	**Yes**	**60 / 38**	**46 / 30**	**34 / 24**	**35 / 18**
Osprey	osp	No	No	52 / 15	44 / 21	33 / 10	30 / 19
Crescent	cre	No	No	50 / 12	36 / 24	38 / 10	34 / 23
Hornet	hor	No	No‡	60 / 27	32 / 23	44 / 15	30 / 24

† A single, male *A. sagrei* was observed on South Twin in 2010 (Y.E.S. personal observation), suggesting that colonization had already started. That individual was captured and euthanized. No *A. sagrei* were observed on South Twin in 2011.

‡ A single, male *A. sagrei* was observed on Hornet Island in 2019. It was captured on the westernmost edge of the island (the closest point to the mainland) and euthanized.

Table 3. Change in *A. carolinensis* perch heights on one-species and two-species control islands from 2010 to 2019. Results from a linear mixed effects model: perch height ~ year x one-versus-two species + sex + (1|island). Here, females from one-species islands in 2010 are the baseline (*i.e.*, the intercept) against which all other categories are compared.

	β (cm)	Std. Err.	D.F.	t-value	p-value
Perch Height Intercept	87.7	11.7	786	7.5	**< 0.001**
Year (2019)	4.1	8.9	786	0.5	0.643
Two-species (Yes)	66.7	15.0	4	4.5	**0.011**
Sex (Male)	27.4	6.8	786	4.0	**<0.001**
Year x two-species	57.6	12.4	786	4.6	**< 0.001**

Table 4. Change in *A. carolinensis* size-corrected toepad area on one-species and two-species control islands from 2010 to 2019. Results from a linear mixed effects model: toepad area ~ year x species category + sex + (1|island). Here, females from one-species islands in 2010 are the baseline (*i.e.*, the intercept) against which all other categories are compared.

	β	Std. Err.	D.F.	t-value	p-value
Intercept	2.52	0.08	593	32.2	**<0.001**
Year (2019)	0.04	0.03	593	1.3	0.207
Two-species (Yes)	0.21	0.11	4	2.0	0.121
Sex (Male)	1.56	0.02	593	61.6	**<0.001**
Year x two-species	0.04	0.05	593	1.0	0.327

Table 5. Change in *A. carolinensis* lamella number on two-species and one-species control islands from 2010 to 2019. Results from a linear mixed effects model: lamella number ~ year x one-versus-two species + sex + (1|island). Here, females from one-species islands in 2010 are the baseline (*i.e.*, the intercept) against which all other categories are compared.

	β	Std. Err.	D.F.	t-value	p-value
Intercept	22.70	0.21	593	107.8	**<0.001**
Year (2019)	0.11	0.11	593	0.97	0.334
Two-species (Yes)	0.49	0.28	4	1.76	0.154
Sex (Male)	1.29	0.09	593	14.55	**<0.001**
Year x two-species	-0.22	0.16	593	-1.38	0.168

Table 6. *A. carolinensis* perch heights changed from 2010 to 2019 on five islands. Results

from a linear model: perch height ~ year x island + sex. Adding the coefficient for the effect of 2019 to the island x year interaction coefficient gives the estimated change in perch height from 2010 to 2019 for a given island (final column). Here, females from one-species islands in 2010 are the baseline (*i.e.*, the intercept) against which all other categories are compared.

	β (cm)	Std. Err.	t-value	p-value	Estimated within island change
Perch Height Intercept	100.3	9.6	10.4	**<0.001**	NA
Year (2019)	53.9	13.4	4.0	**<0.001**	53.9†
Sex (Male)	31.7	13.7	5.2	**<0.001**	NA
Channel Island (two species)	71.6	14.3	5.0	**<0.001**	58.5
2019 x Channel	4.6	20.2	0.2	0.82	
North Twin Isl. (two species)	50.4	13.8	3.6	**<0.001**	18.3
2019 x North Twin	-35.6	20.0	-1.8	0.075	
Yang Island (two species)	31.9	13.7	2.3	**0.020**	118.4
2019 x Yang	64.4	21.0	3.1	**0.002**	
Crescent Island (one species)	5.7	14.3	0.4	0.69	-14.6
2019 x Crescent	-68.5	20.8	-3.3	**0.001**	
Hornet Island (one species)	-29.8	12.9	-2.3	**0.021**	30.7
2019 x Hornet	-23.2	20.2	-1.1	0.25	
Osprey Island (one species)	19.2	13.9	-1.4	0.17	-4.4
2019 x Osprey	-58.3	20.3	-2.9	**0.004**	

Model: $F_{14, 954} = 26.7$, $p < 0.0001$, Adj. $R^2 = 0.27$

† This is the estimate of the change from 2010 to 2019 on South Twin, all else equal.

Table 7. Size-corrected toepad area increased on all islands in 2019, relative to 2010. South Twin females in 2010 are the baseline of this model. 'Year x island' change sums the coefficients of the year effect (0.11) and a specific interaction effect for an estimate of island change from 2010 to 2019. Here, females from one-species islands in 2010 are the baseline (*i.e.*, the intercept) against which all other categories are compared.

	β	Std. Err.	t-value	p-value	Estimated within island change
Intercept	2.61	0.039	65.85	**<0.001**	NA
Year (2019) †	0.11	0.054	2.12	**0.034**	0.11†
Channel Island (two species)	0.02	0.054	0.29	0.77	0.10
2019 x Channel	-0.01	0.078	-0.12	0.90	
North Twin Isl. (two species)	0.23	0.06	3.99	**<0.001**	0.00
2019 x North Twin	-0.11	0.08	-1.43	0.15	
Yang Island (two species)	0.12	0.05	2.32	**0.020**	0.15
2019 x Yang	0.03	0.08	0.45	0.65	
Crescent Island (one species)	-0.10	0.06	-1.79	0.074	0.11
2019 x Crescent	-0.01	0.08	-0.09	0.93	
Hornet Island (one species)	0.06	0.05	1.20	0.23	0.02
2019 x Hornet	-0.10	0.08	-1.27	0.20	
Osprey Island (one species)	-0.24	0.06	-4.22	**<0.001**	-0.01
2019 x Osprey	-0.12	0.08	-1.49	0.14	
Sex (Male)	1.56	0.02	68.07	**<0.001**	NA

Model: $F_{14, 697} = 352.5$, $p < 0.0001$, Adj. $R^2 = 0.87$

† This is the estimate of the change from 2010 to 2019 on South Twin, all else equal.

Table 8. Lamella number increased on South Twin in 2019, relative to 2010. South Twin females in 2010 are the baseline of this model. 'Year x island change' sums the coefficients of the year effect (0.38) and interaction effect for an estimate of island specific change from 2010 to 2019. Here, females from one-species islands in 2010 are the baseline (*i.e.*, the intercept) against which all other categories are compared.

	β	Std. Err.	t-value	p-value	Estimated within island change
Intercept	22.51	0.14	158.96	**<0.001**	NA
Year (2019) †	0.38	0.192	1.99	**0.046**	0.38†
Channel Island (two species)	0.20	0.19	1.07	0.28	0.066
2019 x Channel	-0.32	0.28	-1.14	0.25	
North Twin Isl. (two species)	0.80	0.20	-1.59	**<0.001**	-0.120
2019 x North Twin	-0.50	0.28	-1.77	0.078	
Yang Island (two species)	0.94	0.19	5.00	**<0.001**	-0.286
2019 x Yang	-0.67	0.28	-2.40	**0.017**	
Crescent Island (one species)	0.66	0.20	3.31	**0.001**	-0.100
2019 x Crescent	-0.48	0.28	-1.75	0.080	
Hornet Island (one species)	-0.30	0.19	-1.59	0.11	0.494
2019 x Hornet	0.11	0.27	0.40	0.69	
Osprey Island (one species)	0.17	0.20	0.82	0.41	-0.104
2019 x Osprey	-0.49	0.28	-1.71	0.088	
Sex (Male)	1.33	0.08	16.26	**<0.001**	NA

Model: $F_{15, 696} = 24.6$, $p < 0.0001$, Adj. $R^2 = 0.33$

† This is the estimate of the change from 2010 to 2019 on South Twin, all else equal.

Table 9. Nearby *A. sagrei* is correlated with *A. carolinensis* perch height. (A) Results for 2019 linear mixed model with island as a random effect. South Twin as the baseline. (B) Results for 2010 linear mixed model with island as a random effect. Channel is the baseline. South Twin was one-species in 2010.

(A) 2019	Coefficient (cm)	Std. Err.	Deg. Freedom	t-value	p-value
Intercept	186	21.1	261	8.8	**< 0.001**
A. sagrei nearby (Yes)	55	12.6	261	4.3	**< 0.001**
Sex (Male)	8.1	12.2	261	0.66	0.510
(B) 2010					
Intercept	147	16.9	196	8.6	**< 0.001**
A. sagrei nearby (Yes)	-43	17.1	196	-2.4	**0.012**
Sex (Male)	50	17.1	196	2.9	**0.004**

Figure Captions

Figure 1. Mean perch heights for *A. carolinensis*, by island, in 2010 and 2019. Standard errors are shown. Islands are staggered within year. Control islands are in grey. Filled circles are two-species islands. Open circles are one-species islands. The black square is South Twin. Recall that South Twin was a one-species island in 2010, and a two-species island in 2019.

Figure 2. Change from 2010 to 2019 in mean size-corrected toepad area and mean lamella number, by island. Standard errors shown. Lines connect the same island across years. These are raw values; they do not take into account the sex and year x island effects reported in Tables 7 and 8. Control islands are in grey. Filled circles are two-species islands. Open circles are one-species islands. The black square is South Twin.

Figure 3. *A. carolinensis* perch height is positively correlated with mean *A. sagrei* density (β = 3.4cm, $p = 0.0002$). Excluding the one-species islands from the linear regression reveals a similar, positive relationship, but it is no longer significant (β = 3.8cm, $p = 0.08$). Island means are shown with standard errors. Values at $x = 0$ are jittered.

Figure 4. Finding *A. sagrei* nearby is correlated with a significant increase in perch height by *A. carolinensis* in 2019 (panel A; Table 9A) but not 2010 (panel B; Table 9B). Each point is an individual. Males are offset from females. Solid lines connect female means in each *sagrei*-nearby category. Dotted lines connect male means. South Twin has no data in 2010 because it was an *A. carolinensis*-only island at that time.