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Brief Communication

Allochronic isolation between sympatric populations of an alpine butterfly

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Abstract

The role of isolation by distance in shaping population structure across space is well understood, and the same principles should operate over time. Allochrony, the isolation of populations due to separation in time, can be a source of genetic differentiation, but remains infrequently documented between yearly asynchronous populations. In this study, we investigate divergence between 2 putative sympatric populations of the alpine butterfly *Oeneis chryxus ivallda* at Castle Peak, CA, USA. We find clear genetic differentiation between butterflies collected in odd and even years, with limited instances of admixture. The observed G_{ST} of 0.05 between the two populations is approximately equivalent to 26 km based on pairwise G_{ST} values observed between *O. chryxus* populations across space. These results demonstrate a potential source of population differentiation in systems that promote multiple-year development in insects, often found in high-elevation and high-latitude environments.

Keywords: allochrony, sympatry, population structure, genetic drift, butterflies

Introduction

A central principle of evolutionary biology is that populations separated in space, subjected to varied selection pressures and stochastic processes, become differentiated (Rousset, 1997; Wright, 1943). Examples of ecological and evolutionary forces interacting to drive reproductive isolation across space are manifold (Bohonak, 1999; Rundle & Nosil, 2005; Slatkin, 1987); however, these mechanisms can also operate over time (Hendry & Day, 2005). Temporal, or allochronic, isolation can arise from phenological variance within a day, between seasons, or across years, but in each instance, populations separated in time become distinct (Bell et al., 2017; Fudickar et al., 2016; Fukami et al., 2003). The capacity of seasonal allochrony to contribute to divergence is relatively well established (Feder et al., 1988; Friesen et al., 2007; Santos et al., 2007), but the extent to which yearly temporal isolation, in which sympatric populations occur in different years, can generate population structure is less understood (Taylor & Friesen, 2017).

Unlike seasonal separation, where environments vary over time, yearly allochronic cohorts experience largely the same conditions, resulting in fewer opportunities for divergent selection. Despite this, some instances of yearly allochrony promoting large-scale divergence are known, perhaps best exemplified by the highly periodic genus *Magicicada* (Ritchie, 2001; Simon et al., 2022). Cicadas in this genus comprise three distinct species groups across eastern North America, each with species that emerge every 13 or 17 years and, as a result, are almost entirely reproductively isolated, co-occurring only every 221 years (Sota et al., 2013). Multiple hypotheses have been proposed to explain this phenomenon, ranging from temperature during the last glacial maximum (Ito et al., 2015) to predation pressure (Koenig & Liebhold, 2013), both of which suggest that differentiation is driven by environmental drivers. Similar results are seen in Atlantic salmon (*Salmo salar*), where individuals return to their spawning grounds after spending between 1 and 5 years at sea, thought to be a trade-off between survival to reproduction and reproductive output (Hutchings & Jones, 1998). The size and age at which fish return appear to be genetically determined, leading to reproductive isolation and divergence between sympatric populations with differing maturation ages (Johnston et al., 2014). In both systems, cohorts are separated across different years, yet there are clear selective mechanisms that are thought to establish and maintain considerable reproductive isolation.

While these cases demonstrate how selection can lead to substantial yearly allochronic divergence, the capacity for smaller-scale processes to produce temporal differentiation has been less explored, but may be common (Devaux & Lande, 2008). Such allochronic isolation would be expected in environments that promote small, annually separated populations (Berlocher & Feder, 2002; Heliövaara et al., 1994). Such conditions arise in high-elevation and

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high-latitude insects, where cold environments, often also characterized by low primary productivity or low-quality food resources, extend developmental times beyond a single year, allowing for the possibility of nonoverlapping cohorts (Danks, 1992). The pine bark bug (*Aradus cinnamomeus*) in southern Finland offers insight into this process, where two large populations are separated both spatially and by asynchronous 2-year life cycles (Heliövaara & Väisänen, 1987). Each large population has a much smaller sympatric population that emerges in the alternate year (Heliövaara & Väisänen, 1987). These small, off-year populations are genetically distinct from the larger populations they co-occur with in space (Heliövaara et al., 1988). The two large populations, however, separated in space and time, are not genetically distinguishable from each other (Heliövaara et al., 1988), a pattern consistent with drift driving differentiation in the smaller populations. Similar questions have been studied in biennial alpine butterflies: *Erebia euryale* and *Erebia palarica* in Europe (Bouaouina et al., 2023; Vila & Björklund, 2004) and *Oeneis macounii* and *Oeneis melissa semidea* in North America (Gradish et al., 2015, 2019). In *E. euryale* and *O. melissa*, differentiation was detected between allochronic populations (Bouaouina et al., 2023; Gradish et al., 2015), but in *E. palarica* and *O. macounii*, no difference was found (Gradish et al., 2019; Vila & Björklund, 2004). Thus, although multiyear development is relatively widespread among insects in cold environments, the conditions under which it translates into measurable temporal population structure remain unclear.

In this study, we investigate yearly allochronic separation between two putative sympatric populations of the alpine butterfly *Oeneis chryxus ivallda*, collected over 5 consecutive years from Castle Peak in the northern Sierra Nevada Mountains of California. Unlike many other biennial populations, which fluctuate between a high "on" year and low "off" year census population size, long-term monitoring has shown that locality has historically had a stable population size in both odd and even years, suggesting two distinct populations (Halsch et al., 2024; Nice & Shapiro, 2001). Using a reduced-representation sequencing approach (Peterson et al., 2012), we explore whether there are differences between odd- and even-year butterflies collected at the same location. We then examine the admixture and evolutionary histories of both hypothesized populations. Finally, we quantify the magnitude of the observed differentiation by associating temporal with spatial differences. In doing so, we examine populations in an environment where genetic drift is likely the major factor contributing to divergence and explore a potential mechanism underlying differentiation in small, isolated, high-elevation insect populations.

Methods

Study system

Oeneis chryxus (Family: Nymphalidae, Subfamily: Satyrinae) is found in the northeastern United States, southeastern Canada, and above the tree line in montane western North America (Scott, 1986). Within the Sierra Nevada Mountains of California, *O. chryxus* is considered a separate subspecies, *Oeneis chryxus ivallda* (MacDonald et al., 2024; Mead, 1878). Across its range, *O. c. ivallda* is primarily found in open, rocky alpine habitats, where it consumes grasses and sedges as larval host plants (Scott, 1986). It develops over 2 years, and because of this, *O. c. ivallda* is abundant only in odd-numbered years in most locations within the Sierra (Nice & Shapiro, 2001). In one locality, Castle Peak, which has been a location of continuous long-term monitoring for over 50 years (Forister et al., 2010), they were found in high abundance every year.

Data collection, extraction, and sequencing

Between 1989 and 1995, 527 *O. chryxus* individuals were collected from 13 sites across the Sierra Nevada and an additional site in the Great Basin as part of a separate study (Nice & Shapiro, 2001) (Figure 1). This collection effort included 5 consecutive years at Castle Peak, during which 17, 13, 25, 23, and 20 individuals were collected between 1991 and 1995, respectively. A smaller number of individuals were collected from the Sweetwater Mountains in 1992, 1993, and 1995 (50 total individuals, but only 3 from the even year). All individuals were originally processed for allozyme analysis, and homogenates were stored at −80 °C after this initial use.

In 2024, we extracted DNA from the homogenates using the DNeasy Blood and Tissue Kit (Qiagen Inc., Alameda, CA, USA). We then built a reduced representation genomic library for 511 individuals across the Sierra, including 98 from Castle Peak, using previously described methodologies (Gompert et al., 2014; Parchman et al., 2012). DNA was digested with the EcoR1 and Mse1 restriction enzymes and Illumina adaptors, with unique 8- to 10-bp individual multiplex identifier (MID) sequences were ligated to the resulting fragments. We then amplified fragments using two rounds of PCR using iProof high-fidelity polymerase (Bio-Rad Inc.) and pooled the resulting amplicons. Fragments between 300 and 450 bp were selected using a BluePippin (Sage Science Inc., Beverly, MA, USA), and the resulting fragments were sequenced on two lanes of an Illumina Nextseq 2000 (single read, 150bp) at the SUNY Upstate Medical University's Molecular Analysis Core (SUNYMAC, Syracuse, NY).

Bioinformatics

PhiX reads were removed by assembly to the PhiX genome using bowtie version 1.1.2 (Langmead et al., 2009). We then removed the MIDs, short reads, and any reads that contained Mse1 adapter sequence. The remaining reads were written into separate fastq files for each individual (median = 3,861,495 reads per individual). We screened out 79 additional individuals who received fewer than 800,000 total reads. After this, we were left with 432 individuals, including 13, 10, 16, 22, and 17 individuals from Castle Peak from the years 1991–1995, respectively. After screening, all remaining reads were then aligned to an *O. chryxus* genome (MacDonald et al., 2024) using the mem algorithm of bwa version 0.7.18 (Li & Durbin, 2009).

After alignment, we performed variant calling and additional filtering twice: first using all individuals and then only those collected at Castle Peak. In both cases, variable sites were identified using bcftools version 1.9 (Li et al., 2009) using the mpileup and call commands, ignoring indels and retaining variable sites if the posterior probability that the nucleotide was invariant was <0.05. We further filtered this resulting variant call format file using custom scripts where

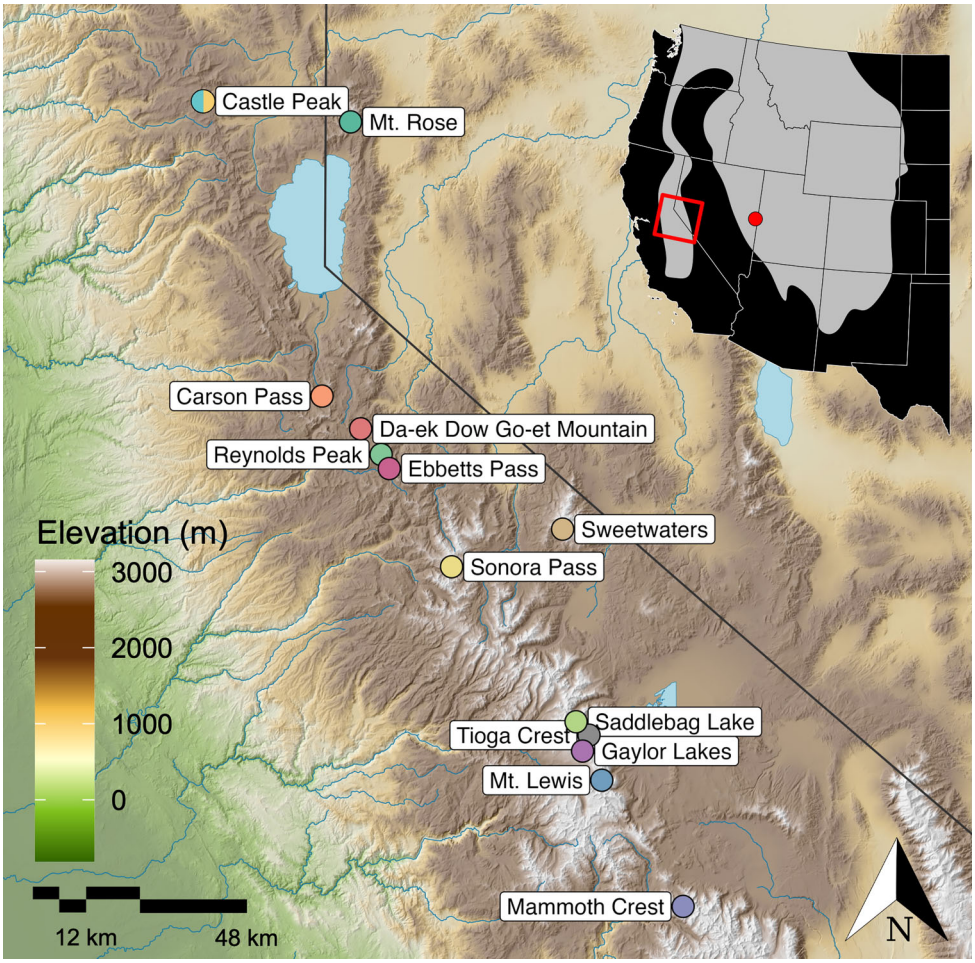

Figure 1. Map of 13 collection localities sampled within the Sierra Nevada mountains in the Western United States between 1991 and 1995. Sites are uniquely colored, which aligns with the full phylogenetic tree shown in Figure S5. Castle Peak is labeled with a multicolored point to indicate hypothesized sympatric populations. Labels are directly horizontal to the point they refer to. The inset map shows the western U.S. distribution of *Oeneis chryxus* in gray and the study area in the Sierra Nevada Mountains of California, USA, outlined in red. One additional locality was sampled in the Snake Mountains of eastern Nevada for use as an outgroup (indicated by the point on the inset map).

variable sites were removed if they had a sequence depth of less than twice the number of individuals, a mapping quality less than 30, an absolute value of the mapping quality rank sum test greater than 1.96, an absolute value of the read position rank sum test greater than 1.96, an absolute value of the base quality rank sum test greater than 1.96, minor allele frequency less than 0.05, or missing data for more than 50% of the individuals. We limited variable sites to one per contig to minimize linkage disequilibrium. These steps resulted in a final dataset of 432 individuals and 27,304 variable sites across all populations and 78 individuals and 21,673 variable sites when only looking at Castle Peak. We explored stricter filtering thresholds, and all inferences remained the same; these are presented in the supplementary materials (Figures S1 and S2).

Statistics

To understand the distribution of genetic variation between butterflies collected in odd versus even years at Castle Peak, we estimated genotypes, allele frequencies, and admixture proportions using the Bayesian admixture algorithm entropy (Gompert et al., 2014; Shastry et al., 2021). We ran this algorithm twice on both the full and Castle Peak-only datasets. For both analyses, we fit multiple models, each specifying a different number of hypothetical ancestral populations: 2–15 for the full dataset and 2–6 for the Castle Peak-only dataset. We also estimated interclass ancestry for the Castle Peak-only dataset using entropy ($k = 2$) with the ancestry-complement model. For each model run, two Markov chain Monte Carlo simulations of 105,000 steps with a burn-in of 5,000 were run, thinning to retain every 10th step. We assessed model convergence by calculating the mean Gelman–Rubin convergence diagnostic for admixture proportions of each individual (Brooks & Gelman, 1998; Gelman & Rubin, 1992) for each chain with the package coda version 0.19-1 in R (Plummer et al., 2006; R Core Team, 2023). We also saved the deviance information criterion (DIC) for each model to use for model comparison.

We investigated evolutionary relationships among individuals by building approximate maximum-likelihood phylogenetic trees with 1,000 bootstrap replicates using FastTree v2.1.11 (Price et al., 2009). Trees were constructed by concatenating single nucleotide polymorphisms (SNPs) from the filtered variant call file generated from the full dataset. This methodology is not suitable for estimating divergence times; however, we were interested only in topology and

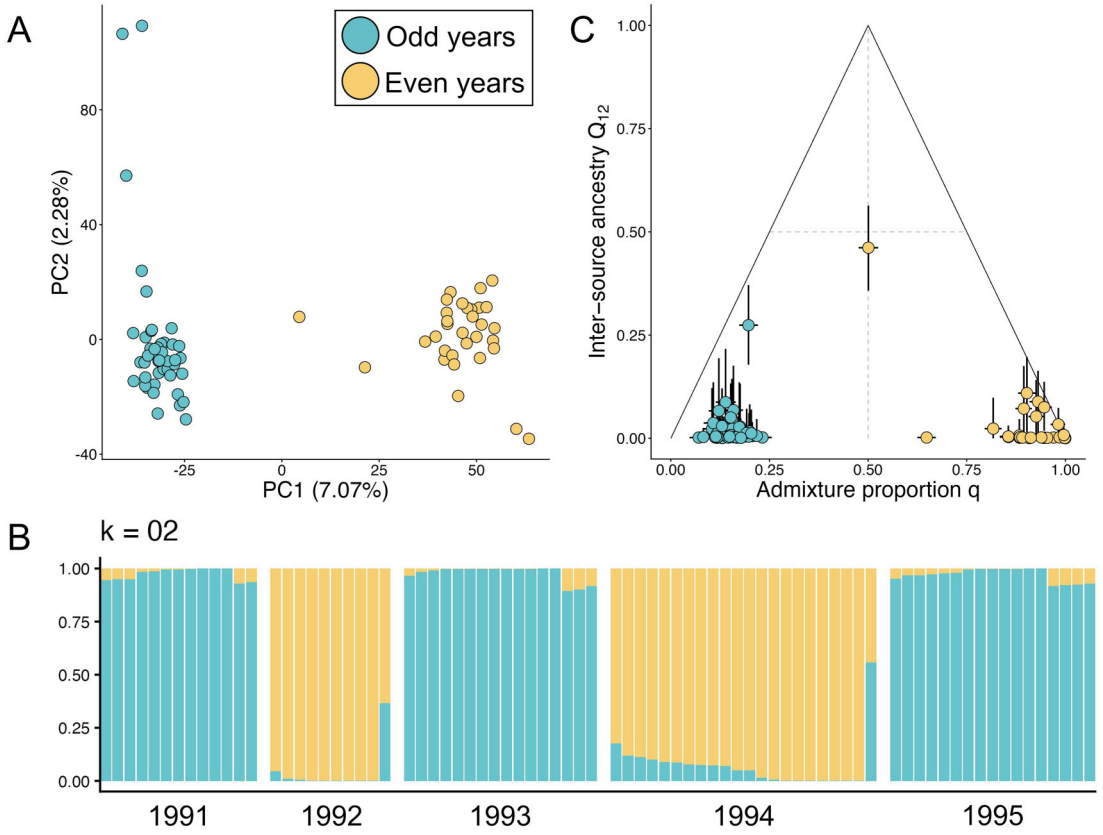

Figure 2. The population structure of *Oeneis chryxus* collected from Castle Peak between 1991 and 1995. (A) Principal component analysis of posterior genotype probabilities summarizing variation across 78 individuals using 21,673 loci. (B) Admixture proportions from entropy models with two hypothesized ancestral populations ($k = 2$) organized by collection year (results for $k = 3$–6 can be found in Figure S4). (C) Results from the ancestry complement model in entropy, where the x-axis is genome-average ancestry and the y-axis is the proportion of sampled loci that are heterozygous for ancestry.

made no inferential statements about the timing of population differentiation. The tree was visualized with the ggtree package in R (Yu et al., 2017).

Genetic diversity was estimated for each site–collection year combination by calculating expected heterozygosity, the Watterson estimator (θ_w) (Watterson, 1975), and nucleotide diversity (θ_π) (Nei & Li, 1979). The Watterson estimator and nucleotide diversity were estimated using ANGSD v0.940 (Korneliussen et al., 2014). For each metric, the average was calculated across individuals for each site–collection–year combination.

To quantify the magnitude of differentiation between collection sites, we calculated genome-average pairwise Nei's G_{ST} (Nei, 1973). This was calculated as the average across all loci and all pairwise site–year combinations, calculated as (average H_t − H_s)/(average H_t). To examine the relationship between G_{ST} and geographic distance, we used a subset of the full G_{ST} matrix by removing all even-year G_{ST} comparisons for sites with sampling in both odd and even years. By removing even-year samples, we compared genetic distances across space alone, removing any additional differentiation due to time. We then linearized values using $G_{ST}/(1 - G_{ST})$ and performed a robust linear regression after using the MASS package (Venables & Ripley, 2002) to quantify the relationship between G_{ST} and distance, which we used only to obtain an effect size, not for hypothesis testing. To infer differences between sites due to spatial and temporal isolation, we generated confidence intervals (CI) around each population's G_{ST} using 1,000 bootstrap samples and report the mean and 95% CI for each pairwise comparison. For differences over distance, Castle Peak odd years were used as the comparator group, and we examined bootstrapped pairwise G_{ST} values between Castle Peak odd-year samples and all other collection locations. To examine temporal differences, we compared bootstrapped pairwise G_{ST} values for samples collected from the same site in different odd years, different even years, or between odd and even years. All analyses were conducted in R (R Core Team, 2023).

Results

Sequencing produced 1,988,942,122 reads with MIDs for 511 individuals (median = 3,861,495 reads per individual, minimum = 786, maximum = 8,786,952). For analysis of the full dataset (from all collection sites), 191,225,644 reads were retained in the final assembly after filtering, for an SNP set of 27,034 loci. Mean sequence depth was 17.3 reads per individual per locus ($SD = 13.7$). For analysis of the Castle Peak-only dataset, 16,401,409 reads were retained after filtering, yielding an SNP set of 21,673 loci. Mean sequence depth was 19.7 reads per individual per locus ($SD = 11.8$). All models for the Castle Peak-only dataset converged; however, for the full dataset, only models with 10 or fewer clusters converged, and only

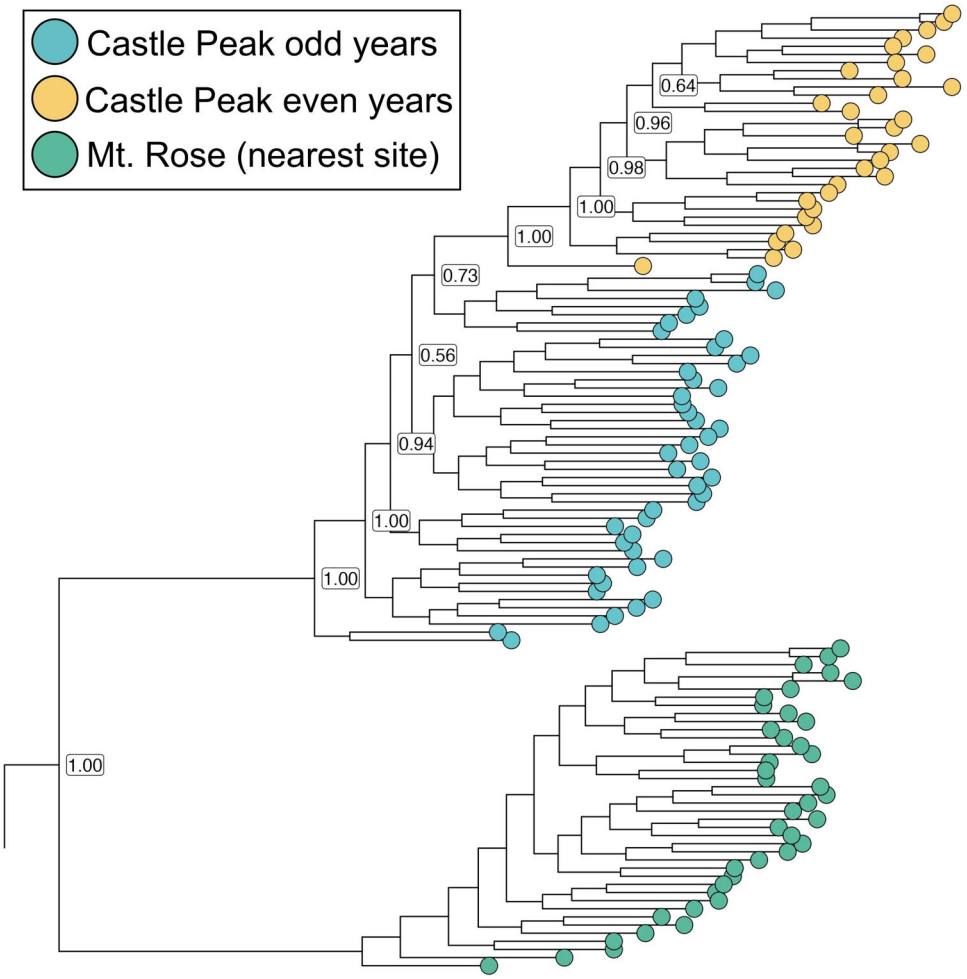

Figure 3. Subset of phylogenetic tree generated from all 14 source populations, showing Castle Peak and the closest geographic site (the full tree can be found in Figure S5). Important nodes for establishing the topology are displayed and labeled with proportional bootstrap values from 1,000 replicates.

results from these were implemented into further analyses (Table S1).

Principal components analysis of the posterior genotype estimates from the Castle Peak-only dataset revealed clear separation between individuals collected in odd and even years (Figure 2A). Differences between years accounted for the greatest axis of variation (PC1, 7% of the variation; Figure 2A). This separation is further supported by results from the admixture analysis, which shows that regardless of the number of hypothesized ancestral populations (k = 2–5 had comparable DIC scores; see Fig. S3), patterns of admixture remain the same. Odd- and even-year individuals are largely distinct, but a few individuals caught in even years show evidence of hybrid ancestry (Figure 2B; Figure S4). This is further explored in the results of the ancestry complement model (Figure 2C), which indicates that one of these individuals, with approximately 0.5 admixture proportion from each population and 0.5 interclass ancestry, represents an F_2 hybrid between even- and odd-year cohorts, while the other hybrid is a later generation backcross (Figure 2C). We did not observe large differences in genetic diversity between the 5 collection years (Table S2).

We then examined the relatedness of the hypothesized populations by constructing a phylogenetic tree using the complete dataset from all 13 collection sites, which represents genetic variation within the Sierra Nevada, and an additional outgroup from the Great Basin (Figure 3; see Fig. S5 for the full tree). The tree topology shows that even-year individuals are derived from odd-year individuals, and the odd-year individuals are paraphyletic with respect to the even-year individuals. Taken together, these results support the hypothesis that the odd- and even-year populations at Castle Peak are at least partially reproductively isolated in time, originated from a single event, and occasionally exchange individuals.

After establishing that odd- and even-year cohorts are genetically distinguishable populations, we quantified the magnitude of differentiation. We did this using pairwise G_{ST} values observed across the full dataset (Tables S3–S5). We observed a strong positive relationship between pairwise distance and G_{ST}, with every 0.1 change in G_{ST} associated with approximately 52 km ($SE \pm 1$ km) (Figure 4A; Table S6). We found that all populations, across space and time, differed from the Castle Peak odd years. While the largest differences were observed between sites that were more spatially distant, we also found differences between temporally separated populations. Specifically, odd and even years at Castle Peak yield an average G_{ST} difference of 0.048 (bootstrap CI: 0.044–0.057) (Figure 4B; Table S3). When

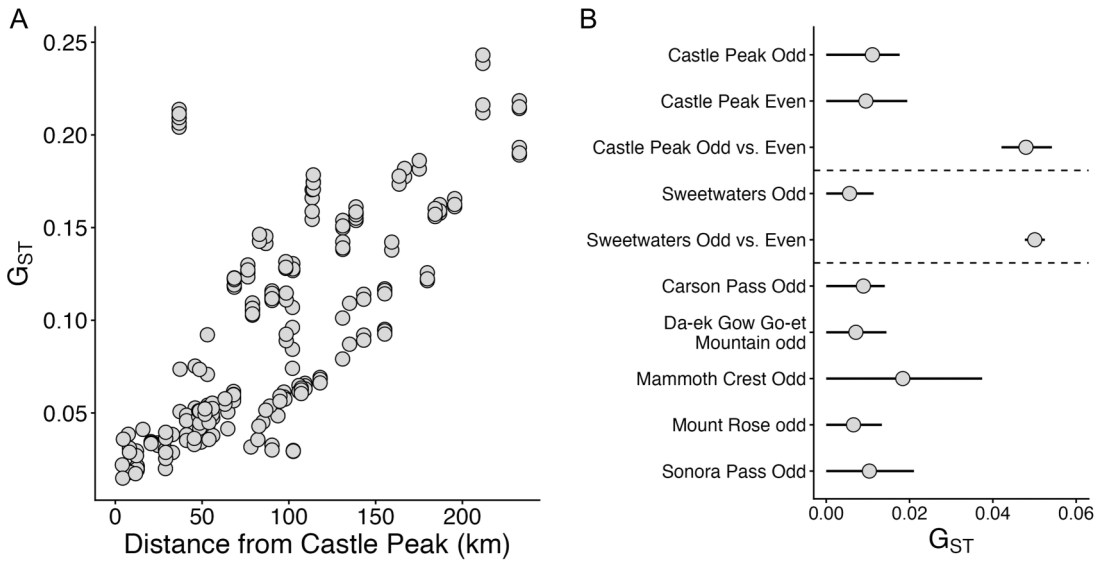

Figure 4. G_{ST} values compared across space and time. (A) G_{ST} values across the 13 collection localities in the Sierra Nevada mountains compared to each of the Castle Peak odd years. (B) Within-site G_{ST} values from sites where multiple samples were taken. Odd indicates that odd years are being compared with other odd years, while odd vs. even indicates that odd years are being compared to even years (from the same site). The confidence intervals around each G_{ST} estimate were generated using 1,000 bootstrap samples.

translated into geographic distance, the average distance between odd- and even-year populations at Castle Peak is approximately equivalent to a geographic distance of 26 km. While the sample size was limited, we also found that the three individuals collected from the Sweetwater mountains in 1992 differed in pairwise G_{ST} by 0.048 (bootstrap CI: 0.048–0.049) and 0.052 (bootstrap CI: 0.051–0.053) from individuals collected in 1993 and 1995, respectively (Table S4).

Discussion

Population divergence due to geographic separation is a classic prediction of evolutionary theory (Wright, 1943); however, in specific systems, a similar pattern can arise from temporal separation (Hendry & Day, 2005). In some cases, allochrony and selection have led to substantial divergence (Taylor & Friesen, 2017), but the frequency and magnitude of differentiation in the absence of strong environmental variation are relatively underexplored phenomena (Devaux & Lande, 2008). Here, we investigated the possibility of allochronic sympatry in the butterfly *Oeneis chryxus ivallda*, a species with a 2-year development cycle that occurred at Castle Peak, CA, every year. We found that individuals collected over 5 consecutive years exhibited clear population structure between odd- and even-year cohorts, with minimal admixture. The observed temporal separation was comparable to 26 km in space. These results provide a clear example of allochronic separation in an extreme environment and raise questions about whether similar processes may occur elsewhere.

Reproductive isolation by time is related to both the temporal distance (relative to the length of the organism's reproductive stage) and the rate of gene flow between different cohorts (Hendry & Day, 2005). Allochronic separation across years has previously been observed in organisms with rigid reproductive cycle timing and where cross-cohort breeding is rare, such as pink salmon (*Oncorhynchus gorbuscha*) and lousewort (*Pedicularis hallaisanensis*) (Aspinwall, 1974; Kim et al., 2024). In contrast, although *O. c. ivallda* typically overwinters twice, it is not obligate, as rearing has shown that both diapauses can be prevented under specific conditions (James & Nunnallee, 2011). Yet, although individuals are not physiologically prevented from emerging in the alternate year, such events appear to be relatively uncommon, as we found only two individuals with evidence of hybrid ancestry among the 78 sequenced individuals. Given this, it seems likely that the environmental conditions necessary to compress development into a single year or extend it to 3 years were, at the very least, unusual.

Low rates of gene flow alone can lead to differentiation via drift in small populations, but consistent environmental differences between populations would further accelerate divergence. In this system, however, such differences are not immediately apparent because both populations share the same location. It is conceivable that climate could vary consistently between odd and even years; however, previous work at this site, examining the impact of weather on butterfly community dynamics, has shown no such pattern (Halsch et al., 2024; Nice et al., 2019). A more probable source of consistent variation between odd- and even-year cohorts is parasitism pressure, where parasitoids follow similar 2-year cycles and attack one population at a higher rate than the other (Kleckova et al., 2015; Várkonyi et al., 2002). Such dynamics have been observed in other alpine butterflies and are postulated to explain differences in census population sizes between odd- and even-year cycles (Wipking & Mengelkoch, 1994). We do not have data on parasitism rates of *O. c. ivallda* in the Sierra Nevada, although Castle Peak is the only site in the Sierra where these butterflies were known to be abundant in both years, so if par-

asitism is a factor in this locality, the pattern of population suppression is different from what has been observed elsewhere (Wipking & Mengelkoch, 1994). Finally, we cannot rule out the possibility that an unexpected event, such as a selective sweep, occurred in one population but not the other, generating the observed differentiation; however, given the limited observed admixture, the isolation of Castle Peak, and the modest observed G_{ST} between populations, the most parsimonious explanation for divergence is genetic drift.

While these allochronic populations of *O. chryxus* offer insight into a mechanism of small-scale differentiation in sympatry, they also stand out as exceptions in the Sierra Nevada. In most locations, this species is primarily observed in odd years, with only occasional observations in even years. This raises the possibility that Castle Peak is better suited than other sites to the establishment of an even-year population from the odd-year population, as suggested by the phylogenetic tree topology (Figure 3). Castle Peak was both the northernmost known *O. c. ivallda* population in the Sierra Nevada and among the lowest in elevation, and as a result, has a comparatively longer growing season. It may be that incipient off-year populations arise more frequently at places like this, at range edges, where exposure to more extreme conditions (relative to the core range) increases developmental variation and, consequently, the likelihood of switching events. In such a place, single or repeated founding events with recurrent low-level gene flow between populations would be more frequent and could generate the observed patterns of ancestry. Of course, Castle Peak is only a single location, and the possibility remains that the distribution of where off-year populations establish and persist may be random, or at least appear to be, even if abiotic and biotic processes are acting at fine scales.

The *Oeneis chryxus ivallda* of Castle Peak illustrate a case in which populations were structured by time, comparable to patterns observed over short geographic distances, despite no known major environmental differences. While the comparable census population sizes between odd- and even-year populations of Castle Peak may indeed have been the exception across the range, the few even-year individuals sequenced from the Sweetwater mountains also exhibited similar differentiation from the sympatric odd-year cohort (Figure 4). The butterflies at that site presented the pattern more typical of *O. c. ivallda* across much of the Sierra Nevada (and other alpine butterflies elsewhere in the world): a large, on-year cohort with infrequently observed off-year individuals (MacDonald et al., 2024). Given the observed differentiation, it remains possible that smaller but still differentiated populations of *O. c. ivallda* may be widespread, as demonstrated in other insects (Heliövaara et al., 1988). Further still, while this and similar work have focused on cold settings (Gradish et al., 2015, 2019; Heliövaara et al., 1988; Vila & Björklund, 2004), insect development times extending over years are a feature of many extreme environments, including those in temperate hot and dry regions as well as in the tropics (Danks, 2013). Even within the *Oeneis* genus, other lower-elevation species are believed to have biennial development (Scott, 1986), and such systems may provide key insights into the frequency and variation of yearly allochronic divergence in extreme environments.

Supplementary material

Supplementary material is available at *Evolution*.

Data availability

Data and code to replicate G_{ST} analyses have been uploaded to figshare and can be found at the following DOI: 10.6084/m9.figshare.30758699

Author contributions

C.A.H.: methodology, formal analysis, investigation, data curation, writing – original draft, and visualization; C.C.N.: conceptualization, methodology, investigation, data curation, writing – review & editing, and supervision; K.L.B.: methodology and writing – review & editing; J.A.F.: methodology and writing – review & editing; M.L.F.: writing – review & editing; G.G.-C.: writing – review & editing; A.M.S.: writing – review & editing; and E.M.G: writing – review & editing, supervision, and funding acquisition.

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Conflicts of interest

The authors declare no conflict of interest.

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