Allochronic isolation on a latitudinal gradient in the polyphagous moth *Hyphantria cunea*

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Abstract

Allochronic speciation, where reproductive isolation between populations of a species is facilitated by a difference in reproductive timing, depends on the existence of seasonality. Seasonality is strongly driven by latitude, so there may be a relationship between latitude and divergence among populations separated by life history timing. Here we explore the relationship between allochronic speciation and latitude using *Hyphantria cunea* (the fall webworm), a Lepidopteran defoliator with red and black colour morphs that may be undergoing an incipient allochronic speciation. We annotated >9000 community science observations of fall webworm to model colour morph phenology and differences in phenotype across North America. We also examined the physiology of two sympatric populations to determine differences in diapause intensity. We found the fall webworm is multivoltine with differing numbers of generations between colour morphs at lower latitudes, and univoltine at latitudes higher than 41°. This shift to univoltism correlated with a decline in morphological differentiation. This shows that conditions at lower latitudes facilitate greater divergence in an incipient allochronic speciation potentially due to longer reproductive seasons allowing for greater mismatches in generations. Our results demonstrate how latitude affects allochronic speciation, and how sympatric speciation along latitudinal gradients contributes to trends in global biodiversity.

Keywords: allochronic speciation, voltinism, community science, macroecology, latitudinal biodiversity gradient, *Hyphantria cunea*

Introduction

Speciation occurs when barriers to interbreeding evolve between members of a species. This can occur in two contrasting ways: allopatric speciation, where new species arise due to geographic isolation between populations, or sympatric speciation, where new species arise without geographic isolation [1]. While it is relatively straightforward to explain reproductive isolation due to allopatric speciation, sympatric speciation requires more complex explanations. Sympatric speciation often has an ecological component leading to reproductive isolation such as differences in abiotic tolerance, host plant use, or other changes to the niche of populations [2]. However, the presence of one of these shifts does not necessitate a speciation event, as the magnitude of the ecological differences can determine the extent of divergence between populations undergoing speciation. For example, the magnitude of host plant shifts is positively correlated with the incidence of speciation in Australian thrips [3,4]. These ecological shifts are not static and often depend on abiotic and biotic factors that vary in space and time.

Sympatric speciation can also occur due to offsets in life history timing between populations, formally known as allochronic speciation. These offsets may lead to the non-overlap of reproductive forms, or the formation of hybrids between populations that are poorly temporally adapted to their environment (e.g. larvae that do not enter diapause in time for winter) [5]. This type of speciation occurs in a variety of taxa, including insects [5–7]. For insects, seasonality and abiotic tolerance play key roles in allochronic speciation. For example, *Rhagoletis pomonella* (the apple-maggot fly) experienced a host-plant shift with the introduction of apple trees to North America, which fruited earlier than the native hawthorn. This non-overlap of host plant

phenology imposed a temporal reproductive barrier between *R. pomonella* host plant races, leading to allochronic speciation [8]. Other previous studies indicate that the ability of an insect population to reduce its diapause duration and increase its voltinism (or number of generations per year) is key to its genetic divergence from other populations [9,10]. This suggests that allochronic speciation in insects may be more effective at higher latitudes where diapause timing is essential to surviving harsher winters. Conversely, it may be less effective at higher latitudes due to shorter breeding seasons preventing increases in voltinism in a population. Phytophagous insects have been suggested as good candidates to study sympatric speciation and the effects of climate and latitude on this phenomenon, as the exceptional diversity of this group has been attributed to a high incidence of sympatric speciation [11,12].

Though allochronic speciation occurs in temporally separated populations that occur together geographically, the progress of this speciation can still be impacted by geographic or latitudinal effects. One of the most general rules in biogeography is that biodiversity is higher in tropical regions for the majority of taxa, possibly due to greater niche specialization and higher rate of molecular evolution in warmer climates [11,13,14]. This has led many to conclude that speciation rates may be higher closer to the equator, and so Mittelbach et al. [11] postulates that divergence among populations of a species should parallel the latitudinal gradient of biodiversity. Several studies have supported this [15,16], including a review of 76 vertebrate and invertebrate species that found within-species diversity was negatively correlated with latitude [17]. Allochronic speciation may similarly be more effective at lower latitudes as longer growing

seasons lead to greater temporal niche space and therefore the potential for greater niche specialization in time.

Despite this, there is evidence that speciation rates may increase at higher latitudes in particular situations. In a review of 309 bird and mammal sister species, Weir & Schluter [18] found a significant increase in speciation and extinction rates towards the poles. Similarly, longer breeding seasons at lower latitudes may lead to greater overlap between generations of populations, reducing reproductive isolation. This was explored in a study of 57 bird species, which found that greater climatic asynchrony (measured by variation in monthly precipitation) is positively correlated with population divergence [19]. This leads to two contradictory possibilities: allochronic speciation may be more effective at lower latitudes because of greater temporal niche availability, or it may be more effective at higher latitudes due to the greater importance of diapause phenology and climate asynchrony. Here, we use the fall webworm (*Hyphantria cunea*, Lepidoptera: Erebidae*)* to investigate these contrasting hypotheses, and we explore how allochronic speciation proceeds along a latitudinal gradient.

The fall webworm is a widespread generalist moth defoliator in North America, whose gregarious larvae live in conspicuous webs on the branches of bushes and trees [20,21]. The fall webworm appears in discrete generations, ranging from one generation a year (univoltine) in northern regions to up to four or five generations per year (multivoltine) in southern regions [22]. Fall webworm larvae occur in two distinct morphs: a red morph and a black morph which exist sympatrically [23]. The red morph has a distinct red head capsule with a brown, tan, or black

body, and the black morph has a black head capsule with a grey or black body. An intermediate maroon morph exists but is only found in northwestern North America. Little is known about this colour morph's phylogenetic relatedness to the red and black morphs, but it is likely geographically separated from the other morphs by the Rocky Mountains [22]. The red and black morphs have both physiological and behavioural differences [24], and genetic analyses show clear separation of the two groups, suggesting reproductive isolation with little gene flow between them despite significant range overlap [20,22,23,25]. However, the two morphs are sexually compatible and produce viable F1 and F2 offspring in the lab [26,27]. Taken together, these results suggest that the two fall webworm colour morphs are currently undergoing sympatric speciation. The black and red morph moth flights do not occur together, their voltinism differs, and the critical photoperiod for pupal diapause is longer in the black morph form, indicating that allochronic speciation may be responsible for the morph's current reproductive isolation [22,28]. Host plant preference was also found to be an unlikely explanation for this speciation since the fall webworm is extremely polyphagous [20], and the colour morphs share some of the same host plants [24].

Here we use a combination of laboratory science and community science methods to investigate how seasonal timing drives speciation across geographic space. The fall webworm is found over a broad latitudinal range (20-51° N), and is conspicuous due to its large nests, so therefore it is frequently photographed by community scientists. As well, it is extremely polyphagous with 600 known host plant species, which reduces the potential effects of host plant specialization on speciation [25,29]. We tested two competing hypotheses: either that allochronic speciation

facilitates greater differences between groups at higher latitudes due to greater abiotic stress and diapause intensity, or allochronic speciation facilitates greater differences between groups at lower latitudes where there is greater temporal niche space. We find that phenology and morphological analyses point to reduced differences between morphs at higher latitudes, corresponding to the latitude in which populations switch from multivoltinism to univoltinism. We also find differences in diapause physiology between sympatric populations of red and black morph, providing evidence for a current incipient allochronic speciation event.

Methods

Data collection and standardization

We decided to focus on the larval life stage because colour morph is only reliably discernable from larval head capsule, not adult colouration [22]. All data for geographic, phenology, and phenotype measures was sourced from images uploaded to iNaturalist (http://inaturalist.org/), a repository of community-collected observations. We found approximately 15,000 observations of *H. cunea*, of which approximately 11,000 were "research grade" (having two matching independent identifications by users) [30]. We examined all research grade images of *H. cunea* uploaded from 2018 – 2020 because previous years had too little data to be suitable for phenology modelling.

Each photo was reviewed manually by one trained researcher (E.N.B), blinded to collection latitude, to identify the larval colour morph and whether its head capsule was visible. In total, 9,486 iNaturalist observations were reviewed. iNaturalist's built-in feature "Observation Fields" was used by E.N.B. to store information with the photograph being classified such as whether the

head capsule was visible (“Head capsule visible?”) and colour morph (“Colour”). The intermediate maroon morph was classified as red in the “Colour” field [22]. No colour morph can be reliably discerned from photos of nests or moths, so these photographs were not included in categorization or analysis.

All analyses were performed using R version 4.0.5 [31]. The dataset and all associated code are published on the Open Science Framework (https://osf.io/x64gp/?view_only=cb630bc7e24d487a9fdaa1a5682e82cb). Raw data from iNaturalist includes observations from North America, and contains the fields: photo ID, user ID, date and time of observation, longitude and latitude, and the custom Observation Fields “Head Capsule Visible?” and “Colour”. Using the tidyverse package [32], observations were filtered to those with an assigned colour morph and visible head capsule. Only larval populations from the eastern United States and Canada (-100 to 0° longitude) were used in analyses so geographically isolated populations like those in Colorado [25] and northwestern North America, and invasive ranges in Asia and Europe were excluded [22,33]. After filtering, 4,594 observations remained and were used for analysis.

Geography

To determine if the colour morphs were geographically separated from one another in their eastern range, we compared the latitude, longitude, and elevation (calculated using the Elevatr package [34]) distribution of each colour morph in each year (Figure 1). Two-way ANOVA was used to determine if colour morph and the year recorded had a significant effect on latitude,

longitude, and/or elevation. SimpleMappr online software (www.simplemappr.net) was used to generate maps of North American fall webworm distribution.

Phenology

Because phenology is climate-dependent [35] and climate is influenced by latitude, observations were split into groups by latitude (29-33°, 33-37°, 37-41°, 41-45° latitude) for analysis. Observations beyond the most poleward and equatorward 5% of observations (<29°, >45°) were excluded from the bands to remove isolated observations. The data were also grouped by year, as phenology may change even at the same latitude due to changing weather patterns from year to year [36,37].

Fall webworm may be univoltine or multivoltine depending on where in their range they occur. A difference in number of generations between colour morphs generated by a model would provide evidence for allochronic speciation [22]. The number of fall webworm observations was modelled at each latitude band using Local Polynomial Regression Fitting ("loess") of fall webworm observations with confidence intervals using ggplot2 (Figure 2) [38]. Span of the loess smoother (span) was set to 0.25 for some smoothing of week-to-week variation and sensitive characterization of peaks in the data. To eliminate day-to-day variation in observations, observations were aggregated by week and colour morph. Since the number of black morph observations was much greater than the number of red and since the relative density of colour morph observations over the year is more important than raw counts, observations/week were scaled from 0 to 1 for each colour morph. All graphics were generated using the ggplot2 package

[38]. To determine the number of generations for each colour morph at each latitude band in each year, we used the algorithm described by Huber [39] to identify local maxima in fall webworm occurrence. For the algorithm, half-width of the window for the local maxima (w) was set to 2, while span of the loess smoother (span) was set to 0.25 (same as loess smoother used in plotting). These two parameters were chosen because they smooth noise in the data, but still allow for sensitive detection of local maxima. Local maxima (or generations) were indicated on the loess modelling of fall webworm abundance (Figure 2). Laboratory-raised larvae pupate 41-42 days after hatching, so time between larval generations predicted by the loess smoother should match or exceed this time [20]. As well, the reproductive form (moths) does not co-occur with larvae, so differences in timing of fall webworm larval generations is a predictor of differences in moth generations [20].

Sympatric population physiology

We collected nests of *H. cunea* larvae from several different locations and host plants in Oklahoma City, Oklahoma, and Norman, Oklahoma during August and September of 2016, which were reared to the pupal stage (rearing methods can be found in Supplemental Methods). Pupae were sampled at three timepoints through the winter and spring: February, March, and May. We prepared pupae for high performance liquid chromatography following the methods of MacMillan et al. [40]. Briefly, pupae were homogenized in 3:1:1 chloroform:methanol:water (6.35 µl per mg tissue), placed at -20 °C for 60 minutes to precipitate protein, then centrifuged at 16,000 RCF to remove debris. The supernatants were then stored at -80 °C. Samples were transferred to the University of Oklahoma Mass Spectrometry Facility where 5 µl of each sample

was injected in technical duplicate on an Agilent 1290 Infinity UPLC system coupled to an Agilent 6545 quadrupole time-of-flight mass spectrometer (QTOF-MS; Agilent, Santa Clara, CA, USA) equipped with a dual-spray ESI source. An Acquity UPLC HSS C18 SB column was used for separation, with solvent gradient from 95% water (plus 0.1% formic acid) to 100% acetonitrile (plus 0.1% formic acid) at a flow rate of 0.4 ml min^{-1}. Raw MS data were then processed as in [41]. XCMS Centwave [42] was used to detect peaks, and mzMatch [43] was used for peak alignment and filtering. Detected features were then matched against metabolites in the Kyoto Encyclopedia of Genes and Genomes (KEGG) [44], LIPID MAPS [45], and HMDB [46] databases with the criterion that the experimentally determined exact mass should be within 6 ppm of the theoretical mass of the metabolite. We conducted all downstream analyses on the average of the two technical replicates. We filtered out any metabolites with a within-group relative standard deviation of >0.5. Using MetaboAnalyst, we then filtered non-informative (relatively constant) metabolites based on interquartile range. We scaled all metabolites by mean-centering and dividing by the standard deviation of each variable to follow assumptions of normality. Finally, we conducted a spare partial least squares discriminant analysis (sPLS-DA) [47] with 10 variables per component to examine the most informative components.

Colour morphology and phenotype

To determine if there is phenotypic divergence between the black and red colour morphs across latitude, we analysed colour data from iNaturalist images. From the standardized dataset containing 4,594 images, those that were low resolution, had poor exposure, were taken from a far distance, or had an object obstructing the webworm were excluded (Figure S3D), leaving

3,789 images for analysis. The colour data was extracted using Adobe Photoshop 2021. Individual larvae were selected from each image using Photoshop's object detection tool (Figure S3A). If Photoshop's object detection tool was unable to properly select the webworm, the freehand lasso tool was used instead (Figure S3B, C). Also, only observations between 29° and 45° latitude and east of -100° longitude were used. Red, green, and blue (RGB) histogram data was extracted from webworm selections as a csv. file using Photoshop's "Record Measurement" feature.

To analyse the colour data, we measured the mean, standard deviation, minimum value, maximum value, kurtosis, and skewness of each RGB histogram. These variables were then normalised (i.e. centered and scaled) [48] in preparation for linear discriminant analysis (LDA) [54]. LDA was used to find a variable that best separated the red and black colour morphs based on the RGB histogram data (Table S1). This variable was named "LD1". To determine if the colour (LD1) of each colour morph depends on latitude, we performed a one-way ANCOVA. We also performed two simple linear regressions to determine the effect of latitude on the colour of each colour morph individually (Figure 4).

Results

Differences in geographic distribution between morphs

Fall webworm populations were expected to occur sympatrically in their Eastern range [22,25], consistent with a sympatric mode of speciation. In our map, red and black morph larvae occurred together across much of their Eastern range. Red/maroon larvae in Colorado and northwestern North America were completely isolated from Eastern populations (Figure 1, Figure S1) [22].

While the two morphs are generally found sympatrically, differential abiotic tolerance may change colour morph distribution within their range. We found a significant difference between the latitudinal range of the morphs ($F_{1,4578}$=113.299, P<0.0001) with black morph populations found an average of 2.4 degrees further north than the red morphs (Figure 1), which interacted significantly with year ($F_{2,4578}$=8.046, P<0.001). We also found a significant difference in the longitudinal ranges of the morphs ($F_{1,4578}$=170.317, P<0.0001) with the red morph populations found an average of 5.8 degrees further west than the black morphs (Figure 1), with no interaction with year ($F_{2,4578}$=0.379, P=0.68). The red morph was found at an average of 32m higher elevation than the black morph ($F_{1,4578}$ =20.345, P<0.0001, Figure S5) with no interaction with year ($F_{2,4578}$=0.128, P=0.88), however a Tukey-Kramer post-hoc analysis found that the elevation difference was not significant in each year (Figure S5).

Differences in phenology between morphs across latitudes

We tested for differences in emergence and last observance dates, which would indicate a difference in phenology. Previous studies show the black morph appears earlier than the red and remains later in the fall [22]. Across all years and latitudes, we found the black larvae appear on average 30 ± 16 days earlier than the red (Wilcox two-sided, P<0.001) and remain 10 ± 9 days later (Wilcox two-sided, P<0.01) when accounting for differences in sample size (see Supplemental Methods, Figure S2).

Using loess smoothing of fall webworm observations and a local maxima-detecting algorithm, we found that fall webworm voltinism varies with latitude and by colour morph, with the number of yearly generations in both red and black morphs negatively correlated with latitude (Figure 2).

Generations of each colour morph in each year are indicated in Figure 2 as solid points on the smoothed lines. At the highest latitudes studied (41-45°), red and black fall webworms are univoltine with a single larval generation. This is the only latitudinal band where the number of generations in the red and black match in each year. From 37-41°, the black morph is bivoltine and the red morph is univoltine. From 33-37°, the trends in voltinism are less clear. The black morph is univoltine in 2018, bivoltine in 2020, and trivoltine in 2019. The red morph is generally bivoltine, however in 2019 they appear to be trivoltine. This may be because these latitudes represent a region where the fall webworm shifts from bivoltine to a trivoltine or greater lifecycle, or phenotypic plasticity leads to variation in voltinism depending on the climate of a particular year [50]. At the lowest latitudes (29-33°), both the red and black morphs have three or four generations, depending on the year, with the red morph being mostly trivoltine and the black morph mostly having four generations.

Differences in physiology of sympatric populations

We found that larvae collected from a common locale in Oklahoma and kept in a common laboratory garden had a difference of approximately 18 days in the onset of pupation between the black and red colour morphs, with the red colour morph beginning pupation on Sept. 25, and the black colour morph beginning October 9 ($F_{1,1959}$=472.91, P<0.001; Figure 3A). We also found a difference of approximately three weeks in the last pupation occurrence between the black and red colour morph, with the last pupation in the red colour morph occurring on Nov. 6, and the last pupation occurring in the black colour morph on Nov. 27. The black morph pupae were significantly heavier than red morph pupae and change in mass over winter was also significantly different between colour morph ($F_{1,1674}$= 21.69, P<0.001) and sex ($F_{2,1674}$= 117.53, P<0.001;

Figure 3B). Pupal mass decreased over time for both colour morphs, with the highest mass loss occurring between November-December and March-April. Due to differential pupation dates found between the colour morphs, with onset of pupation being in September, and the final pupation date occurring in October, change in mass was plotted starting in November.

The HPLC-MS found 2858 peaks, with 712 commonly identified or accepted molecules. Of these, 258 were peptides, 250 were lipids, and 204 were metabolites. Metaboanalyst defined three significant components, with Component 1 (31.6% of the variation) describing change in metabolite content over time, while Component 2 (10.7% of the variation) describing differences in metabolite content between the colour morphs in February (Figure 3C). Metabolites that loaded heavily on Component 1 included N-methylethanolamine phosphate, the peptides Lys-Thr-His and Arg-Lys-Asp-Pro, urate, and betaine aldehyde, while metabolites that loaded heavily on Component 2 included PI(36:4), and the peptides Gln-Phe-Pro-Tyr, Asp-Asp-Gln-His, and Arg-Lys-Met-Val (Table S2).

Differences in colour morphology of morphs across their range

If there are differences in the effectiveness of current allochronic speciation across latitudes, we would expect to see differences in colour morphology across latitude. We next measured colour data from 3,789 webworms extracted from iNaturalist photos to assess morphological differences across latitudes. Of these photos, 3,374 were of black morph webworms while 415 were of red morph webworms. To test the relationship between colour and latitude, we used an LDA model to create a single summary variable (LD1) that best separated the red and black morphs. Among

the coefficients of linear discriminants, the mean green value, mean red value, and red standard deviation had the highest absolute values (-2.73, 1.87, and -1.18 respectively) (Table S1).

We found that the relationship between colour (LD1) of fall webworms and colour morph (red or black) depended significantly on the latitude of the observation (ANCOVA, $F_{1,3525} = 5.187$, $P < 0.05$). Within each colour morph, red morph webworms do not experience any significant colour change across latitude (linear regression, $F_{1,401} = 0.039$, $P > 0.05$, $R^2 = -0.002$) while black morph webworms did experience significant colour change across latitude (linear regression, $F_{1,3126} = 38.49$, $P < 0.001$, $R^2 = 0.012$). The colour of black-headed webworms diverges from red-headed redworms as latitude decreases (Figure 4).

Discussion

Here we show that in the fall webworm allochronic speciation may operate more effectively at lower latitudes where there is greater temporal niche space. In our modelling of fall webworm phenology, we show that red and black morph larvae differ in the number of their generations, except at the highest latitudes (41-45°) where both populations are univoltine (Figure 2). Morphological divergence between red and black morph colour phenotype also rapidly declines past the >41° threshold (Figure 4). The correlation of decline in phenotypic divergence with matching voltinism of red and black larvae at the highest latitudes suggests that conditions at higher latitudes may decrease the effectiveness of allochronic speciation. Finally, we found differences in pupation date and pupal mass between the red and black morphs in sympatry that

may affect their diapause duration and life history timing, providing physiological evidence for a current allochronic speciation event (Figure 3).

Assuming larval emergence is a good predictor of moth emergence, a mismatch in number of red and black morphs generations indicates times where their reproductive forms do not co-occur, facilitating allochronic speciation. This is especially relevant at the beginning of their reproductive season. Prior to the first larval generation, moths emerge post-pupal diapause, mate, and deposit eggs. In regions where black and red morph voltinism does not match and the black morph predates the red, it is likely the black morph moths will appear, mate, and complete a generation before red morph moths have emerged from diapause. So, their chances of encountering each other and interbreeding during this time is low. This would decrease genetic exchange between the red and black morphs and suggests a mechanism for a current allochronic speciation. Also, a mismatch in red and black morph phenology may lead to hybrids between morphs having a voltinism that makes them poorly temporally adapted to their environment [22]. The lack of well-adapted hybrids would reinforce reproductive isolation between the red and black morphs by limiting the possibility for genetic exchange, suggesting another possible mechanism for a current allochronic speciation. This phenomenon would only be observed where there is a mismatch in voltinism between morphs, i.e. at lower latitudes. These conclusions were supported by phenotypic results showing differences between morphs decreasing with increasing latitude, and physiology analysis demonstrating a measurable difference in diapause intensity and physiology.

Since colour morph is only reliably discernable from larval observations [22], we could not model the phenology of the reproductive form (the moth). Using larval phenology to predict

allochronic speciation assumes that both the red and black morph moths appear in the same fixed interval after their larval occurrence. Timing of the life cycle stages of the morphs may have diverged, causing moth phenology to disagree with the larval phenology presented here. Figure 2 is consistent with phenology modelling of moths presented by Yang et al. [22], except at the highest latitudes where they find univoltine black and red morph moths do not overlap in their generations. The discrepancy between our results and those of Yang et al. [22] at the highest latitudes should be explored, as our interpretation relies on larval occurrence being a reliable indicator of moth phenology.

Community science data lent itself well to our broad-scale analysis of fall webworm phenology and phenotype [51], however the lack of consistency in sampling methods may have caused increased variation in the data. Crowd-sourced community science photographs can be biased towards days and seasons with favourable weather conditions and have high rates of mis-identification due to lack of formal training [52]. To address this, images were manually reviewed for species and colour morph and we used smoothing algorithms to minimize daily variation in observation frequency, however this may not completely remove variation in sampling effort. In future studies using community science data, consideration should be given to how to minimize confounding factors due to community sampling, such as using community science data in tandem with field surveys [53]. However, the accessibility of this data, ease of collection, and usefulness for broad-scale research makes it a promising avenue for data collection.

Our laboratory physiological measures, conducted on sympatric populations in a single geographical area, supported the divergence of the two colour morphs in phenology, metabolome, and body mass. We found that the black colour morph pupated later, at a higher body mass, and was clearly differentiated in metabolite profile earlier in the winter (Figure 3). While we cannot differentiate between host plant effects and evolved differences with these data, the clear delineation between colour morphs in host plant preference also points to significant differences between the two colour morphs [25]. It is possible that host plant preference is driving the other observed changes as in the *Rhagoletis* system [54], but it is also possible that abiotic tolerances are driving host plant preference. Results from Vidal et al. [25] suggest that the latter may be the case, as both morphs are extremely polyphagous despite clear preference for certain host plants. So, specialization for the phenology of one host plant leading to allochronic speciation is unlikely, but possible.

Phenotypic results also showed populations of red and black morph webworm look more similar in northern populations (>41°) than those in the south, which may suggest increased interbreeding between morphs in this region. Interestingly, this shift in colour only appears to happen in the black morph, as red morph webworms do not experience any significant colour shift across their entire range (Figure 4). If the colour shift were a result of increasing melanism in northerly populations, as observed in other ectotherms [55], we would expect to observe this shift in both colour morphs.

These results demonstrate that allochronic speciation is not a uniform phenomenon across latitude and depends on voltinism and seasonality [56]. A similar phenomenon was studied using the interactions between *Rhagoletis mendax,* a *Rhagoletis* taxon infesting *V. arboretum*

(commonly known as the sparkleberry fly), and an undescribed *Rhagoletis* taxon. While allochronic speciation caused the divergence of *Rhagoletis mendax* and the undescribed *Rhagoletis* taxon, this divergence caused the secondary contact of the undescribed *Rhagoletis* taxon and the sparkleberry fly. In this case, allochronic speciation led to the reproductive isolation of two taxa and the secondary contact and hybridization of two other taxa [56]. This supports our results finding that allochronic speciation is not solely a mechanism of genetic divergence between populations, but under certain conditions simultaneously facilitates gene flow between closely related taxa.

These findings may complicate the matter of giving species status to the red and black fall webworm. Previous studies suggest that the morphs should be classified as two different species due to complete reproductive isolation and sufficient genetic divergence [22,23]. However, our results suggest this may not apply across the entire fall webworm range. Whether the decreased variation phenotype at higher latitudes represents secondary contact between two species or gene flow between populations of a single species should be further explored using genetic data. Unfortunately, current genetic data is insufficient to answer this question. Furthermore, these results indicate that evidence for allochronic speciation of a limited geographic scope may not be sufficient to claim speciation is complete across an entire taxon; differences in voltinism and phenology may complicate these claims across a species' range.

Conclusion

In this study we tested two competing hypotheses: the first that allochronic speciation operates more effectively at higher latitudes, or the second that allochronic speciation operates more effectively at lower latitudes. Our analyses of phenology and voltinism, phenotypic differences,

and differences in physiology are consistent with the current allochronic speciation in the fall webworm operating more effectively at lower latitudes where there is greater temporal niche space, as differences in voltinism between the morphs is facilitated by a longer breeding season. Further study is needed as some factors such as limited access to high quality and categorized genetic data, variable sampling methods in community science data, and assumptions regarding the fall webworm life cycle and host plant preference may affect our results. Advances in macroecology and community-sourced data provide an avenue for further study of ecological speciation over large geographic areas. Furthermore, investigating the interplay of latitudinal effects and ecological speciation can change our understanding of how speciation operates under different conditions and help to understand past, present, and future trends in biodiversity. Since the great diversity of phytophagous insects has been attributed to high rates of sympatric speciation, greater exploration of allochronic speciation and other forms of ecological speciation in widespread species could elucidate this evolutionary history [11,12].

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Data Accessibility

All code and raw data are available at:

https://osf.io/x64gp/?view_only=cb630bc7e24d487a9fdaa1a5682e82cb

Competing Interest

The authors have no competing interests to report.

Author's Contribution

Proceedings B is trialling CRediT (Contributor Roles Taxonomy).

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Figure Captions

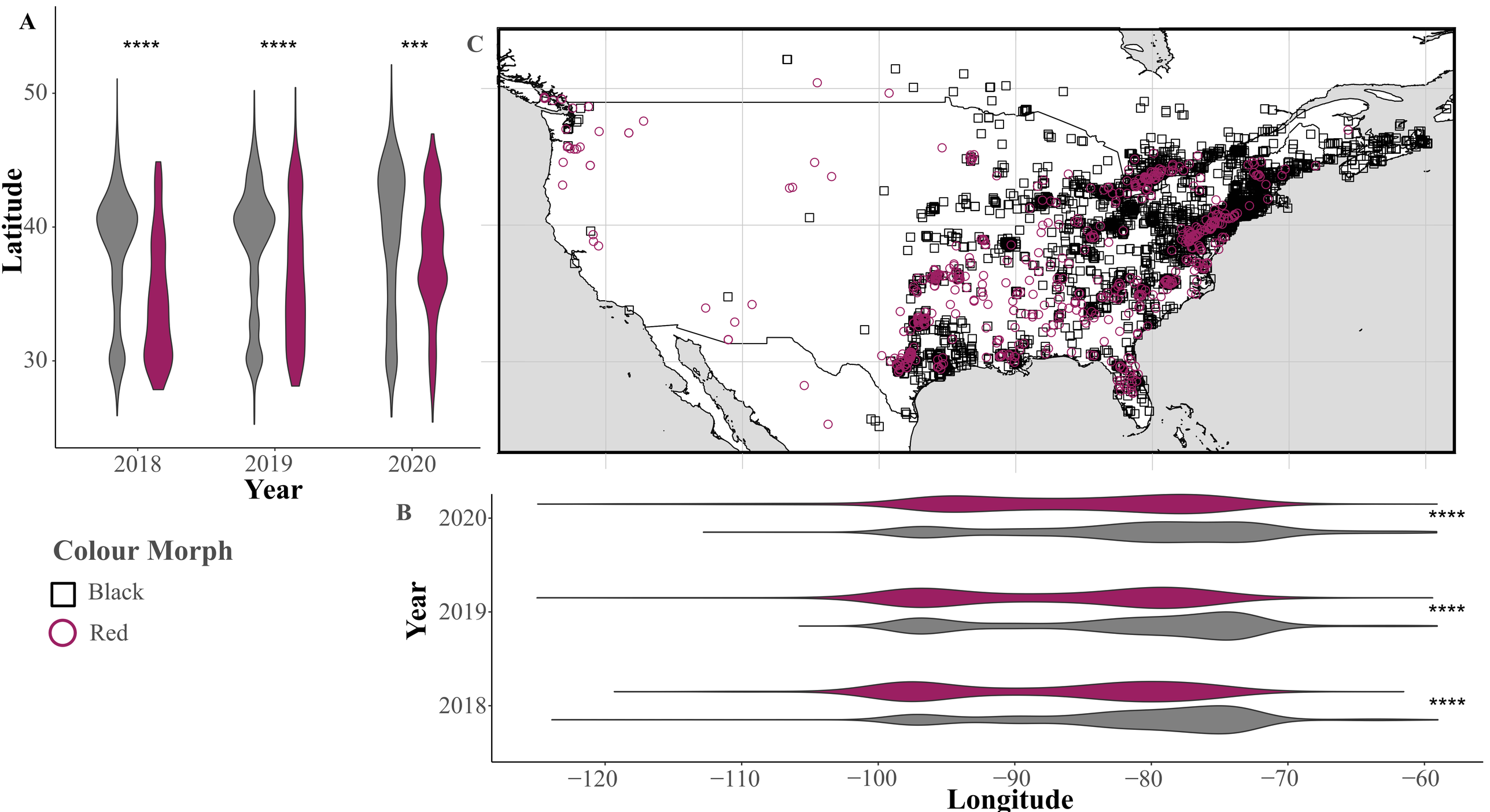
Figure 1. Geographic distribution of North American fall webworm larvae derived from community science observation from 2018-2020. The red and black morphs are found sympatrically (in the same geographic areas), except for isolated red populations in northwestern North America and Colorado. Black morph larvae found an average of 2.4 degrees further north ($F_{2,4578}$=8.046, P<0.001) and 5.8 degrees further east than the red morphs ($F_{1,4578}$=170.317, P<0.0001). A) Latitudinal distribution of black and red morph larvae from 2018-2020. Asterisks showing P-values for differences between each morph each year. B) Longitudinal distribution of black and red morph larvae from 2018-2020. Asterisks represent P-values for differences between each morph each year. C) Map of iNaturalist fall webworm larvae observations in North America from 2018-2020, labelled with colour morph (generated by simplemappr.net). Only larvae where the head capsule was clearly visible are included.

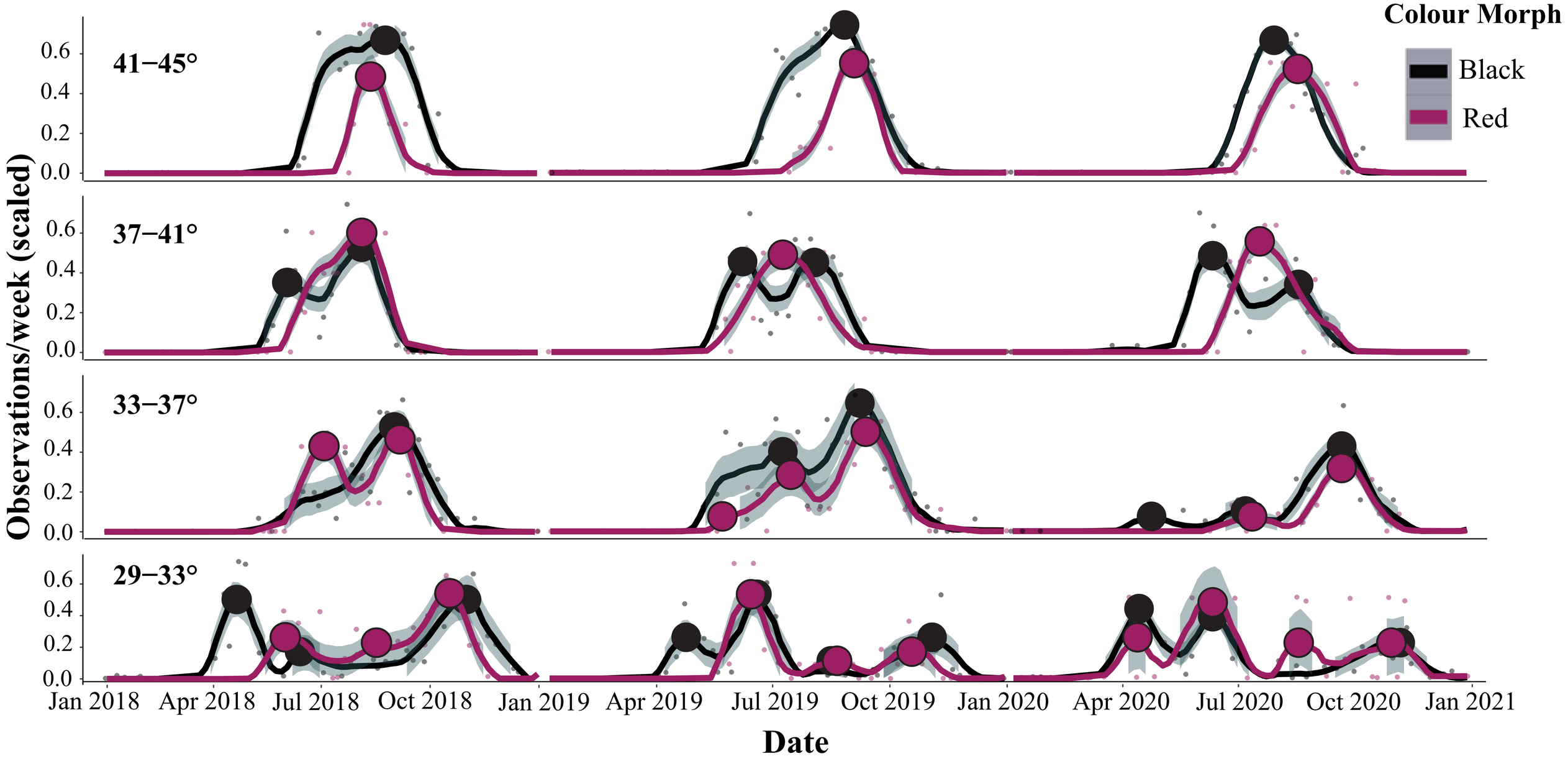
Figure 2. Number of fall webworm observations per colour morph per week (scaled from 0 to 1) from 2018-2020, grouped by latitude. As latitude decreases, the number of generations per year increases in red and black colour morphs, but the number of generations does not match for each morph. Local polynomial regression (loess) fitting shows abundance patterns of each morph over

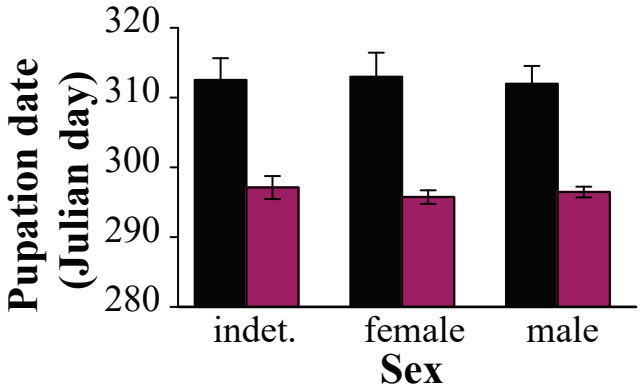
time [38]. Small points represent observations/week for each colour morph. Large circles show the peak of each fall webworm generation. 95% confidence intervals are indicated on loess-fitted curves by blue bars.

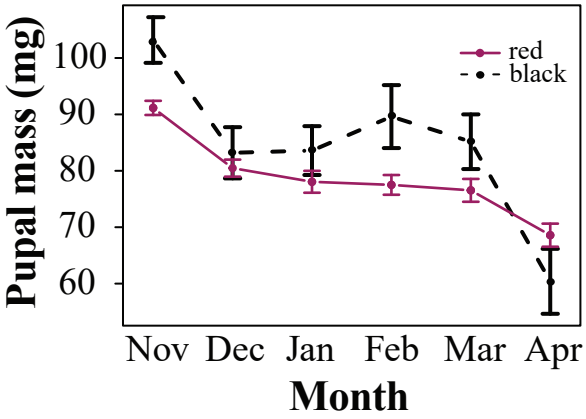
Figure 3. Differences in pupation date and pupal mass between colour morphs in fall webworm populations from Oklahoma. All specimens collected from Norman, Oklahoma in fall of 2016. There were significant differences in pupation date and pupal mass between morphs and sexes, with black morph and female pupae heavier. A) Difference in pupation date between male and female fall webworm pupae, and red and black colour morph larvae. B) Change in overwintering pupal mass of red and black morph fall webworms. C) Principal component analysis (PCA) showing differences in metabolite composition of overwintering red and black fall webworm. Component 1 (31.6% of the variation) describes change in metabolite content over time, while Component 2 (10.7% of the variation) describes differences in metabolite content between the colour morphs in February.

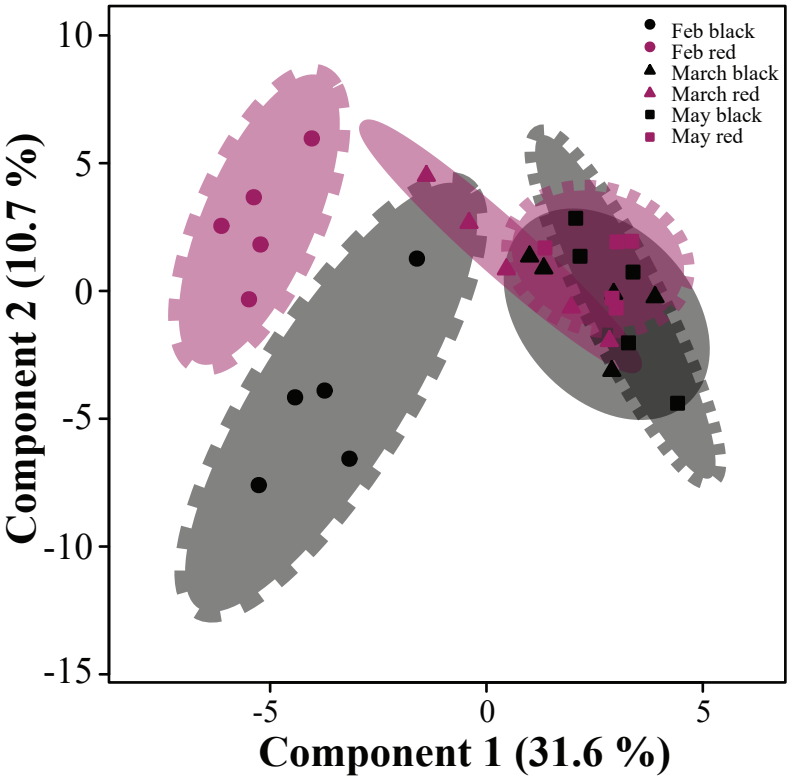
Figure 4. Phenotypic diversity of fall webworm populations across latitude using iNaturalist fall webworm images. LD1 represents the variable that best separates the red and black colour morphs based on image RGB histogram data. Within each colour morph, red-headed webworms do not experience any significant colour change across latitude while black-headed webworms did experience significant colour change across latitude.

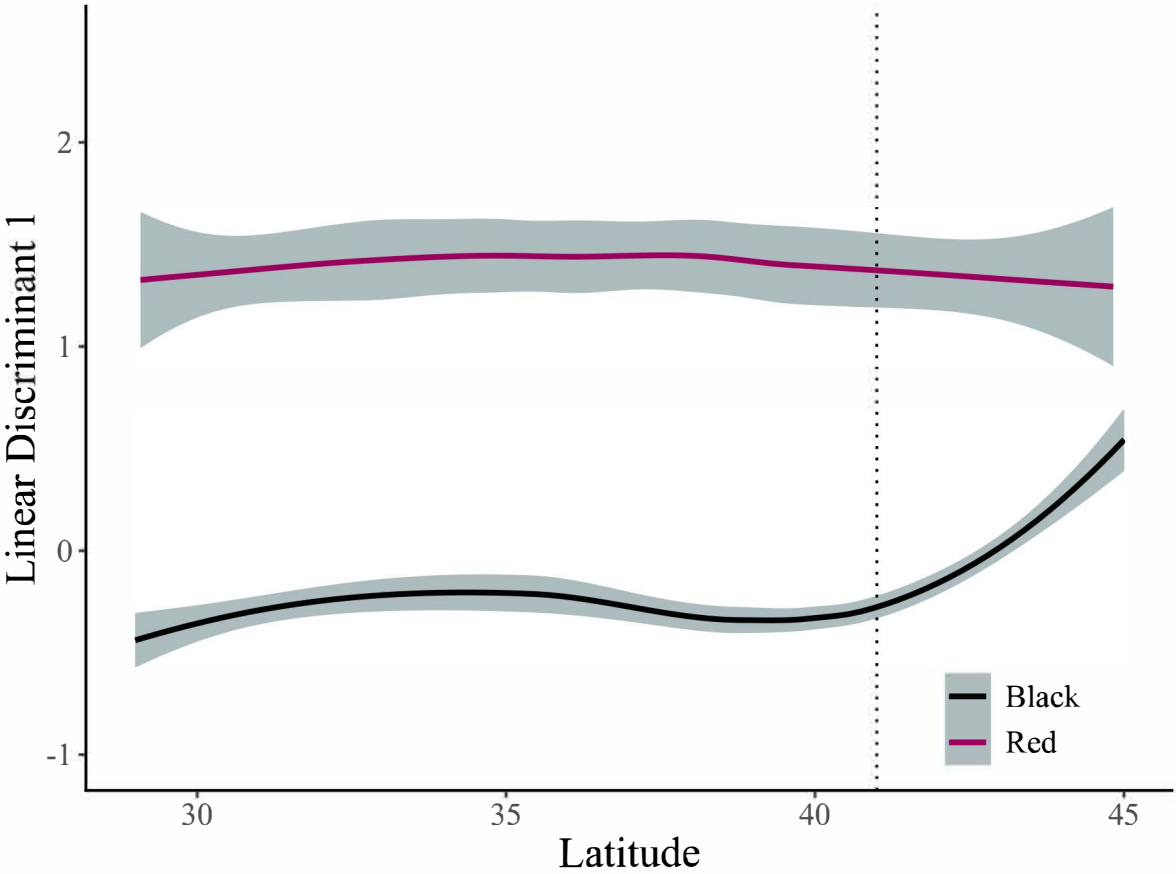

A
****
****
***
50
40
30
Latitude
2018
2019
2020
Year
C
B
2020
2019
2018
Year
****
****
****
−120
−110
−100
−90
−80
−70
−60
Longitude
Colour Morph
Black
Red


Colour Morph
Black
Red
41−45°
37−41°
33−37°
29−33°
0.0
0.2
0.4
0.6
Observations/week (scaled)
Jan 2018
Apr 2018
Jul 2018
Oct 2018
Jan 2019
Apr 2019
Jul 2019
Oct 2019
Jan 2020
Apr 2020
Jul 2020
Oct 2020
Jan 2021
Date


Pupation date
(Julian day)
320
310
300
290
280
indet.
female
male
Sex


Pupal mass (mg)
100
90
80
70
60
red
black
Nov
Dec
Jan
Feb
Mar
Apr
Month


Feb black
Feb red
March black
March red
May black
May red
Component 2 (10.7 %)
10
5
0
-5
-10
-15
-5
0
5
Component 1 (31.6 %)


Linear Discriminant 1
2
1
0
-1
30
35
40
45
Latitude
Black
Red