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Original Article

Anthropogenic increases in nutrients alter sexual selection dynamics: a case study in butterflies

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Anthropogenic increases in nutrient availability offer opportunities to study evolutionary shifts in sexual selection dynamics in real time. A rapid increase in nutrient availability may reduce the utility of condition-dependent ornaments as signals of quality and lessen any nutritional benefits to females from re-mating. We explored these ideas using cabbage white butterflies, focusing on nitrogen as a nutrient, as it is important to this species in not only in ornamentation, but also as a key macronutrient transferred in spermatophores. We compared a nonagricultural population to an agricultural population, which has seen an increase in nitrogen availability over the last 40 years due in part to fertilizer application. When reared in a common garden, both male and female butterflies from the agricultural population allocate significantly more to nitrogen-rich wing pigments used in mate choice. However, this increase in allocation is not correlated with an increase in the ability to assimilate nitrogen from their diet. Spermatophore counts from wild females show that females in the agricultural population rarely mate more than once, while those in the nonagricultural population are much more likely to be polyandrous. Similarly, the structure in the female reproductive tract that processes spermatophores has evolved lower tooth density in the agricultural population, consistent with the idea that these females benefit less from high throughput of spermatophores. These results suggest that anthropogenic increases in nutrient availability have resulted in a decline in the nutritional benefits of rematophores in commentation in both males and females.

Key words: butterfly, mate choice, mating system, nitrogen, sexual selection.

INTRODUCTION

The availability of resources and nutrients plays a prominent role in our understanding of sexual selection. For instance, the utility of sexual traits as indicators of quality stems from the fact that only the highest condition individuals can afford to allocate resources toward a large ornament or costly display (Zahavi 1975; Andersson 1982; Iwasa and Pomiankowski 1991). Those individuals with access to more resources, or with greater ability to acquire, process, and assimilate such resources can afford to allocate resources to displays and ornaments (Rowe and Houle 1996; Tomkins et al. 2004; Hill 2011). Countless studies have used diet manipulations to demonstrate that the expression of sexual traits depends on condition, from carotenoid-based pigmentation (Hill and Montgomerie 1994; Grether et al. 1999; Velando et al. 2006), to courtship displays (Byers et al. 2010) and song (Wagner and Hoback 1999; Buchanan

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et al. 2003; Holzer et al. 2003; Hunt et al. 2004). Similarly, the degree of choosiness of the receiver also depends on condition (Hebets et al. 2008; Fox and Moya-Larano 2009) and in species that receive nutrients from mating, dietary variation plays a prominent role in mating dynamics (Boggs 1990; Leimar et al. 1994). In particular, if females receive direct benefits from mating (Arnqvist and Nilsson 2000), we might expect that increases in nutrition would reduce the relative benefits of choosiness or re-mating (Kaitala and Wiklund 1994; Gwynne 2008; Prokop and Maxwell 2009; Ursprung et al. 2009).

Despite the importance of nutrition in our understanding of sexual selection, we know little about how anthropogenic change in nutrient and resource availability may be altering sexual selection and the evolution of sexual traits (Snell-Rood et al. 2015). In some cases, human activity is directly increasing resource abundance, for instance, through feeding of wildlife or disposal of waste food (Adams 1994; Robb et al. 2008). In addition, nitrogen and phosphorus, macronutrients important in protein, lipid, and nucleic acid synthesis, are increasing in availability due to agricultural

inputs and burning of fossil fuels (Vitousek et al. 1997a, 1997b; Smil 2000; Galloway et al. 2008). An ecological stoichiometric view (Sterner and Elser 2002) allows integration of ideas about condition dependence with the vast ecosystem ecology literature on nutrient cycling (Vitousek et al. 1997a; Smil 2000). While the ecological implications of such anthropogenic nutrient changes are well studied (Tilman 1999; Suding et al. 2005), the consequences for sexual traits and sexual selection dynamics are less clear (Snell-Rood et al. 2015). In the short term, large and sudden increases in nutrient availability may alter sexual selection through decreases in the utility of these traits as indicators of mate quality (Reznick et al. 2000) and lowering the relative benefit of choosing only the highest quality individuals. In the longer term, the utility of these ornaments as indicator traits may be restored if further elaboration increases the relative costs of trait investment (Moller and Pomiankowski 1993; Badyaev 2004). In a sense, anthropogenic environments offer a real-time opportunity to study evolutionary shifts in sexual selection dynamics.

In this study, we explore the hypothesis that anthropogenic changes in nutrient availability alter sexual selection dynamics through changes in both ornamentation and mating patterns. We focus on nitrogen, a major limiting nutrient important in growth, reproduction, and survival (Mattson 1980; Sterner and Elser 2002), which has drastically increased in availability due to human activity. In the United States, the use of synthetic nitrogen fertilizers increased exponentially after World War II: fertilizer inputs from 1961 to 1999 nearly quadrupled from 3.1 to 11.2 million metric tons (Howarth et al. 2002). Additionally, fossil fuel combustion leads to increased atmospheric deposition of nitrogen with many regions seeing an addition of over 10 kg of nitrogen per hectare annually (Dentener et al. 2006).

We use the cabbage white butterfly, Pieris rapae, as our model organism because, like most herbivores, they are limited in growth and reproduction by nitrogen availability (Scriber and Slansky 1981; Sterner and Elser 2002). Furthermore, nitrogen-rich pigments known as pterins are used in mate signaling: females prefer males with higher pterin investment, which is correlated with not only male condition, but also genetic variation in nitrogen assimilation ability (Morehouse and Rutowski In revision; Morehouse and Rutowski 2010; Tigreros 2013). Female pierid butterflies "actively forage" for matings (Rutowski 1980; Kaitala and Wiklund 1994) since spermatophores, which can be up to 15% of a male's body mass with 14% nitrogen content, can increase female fecundity by 50-70 offspring (Watanabe and Ando 1993; Wiklund et al. 1993; Karlsson 1998). Females process spermatophores in the bursa copulatrix, a muscular digestive organ (Sugawara 1979; Meslin et al. 2017), where a toothed structure called the signum breaks open the spermatophore for digestion (Tschudirein and Benz 1990; Galicia et al. 2008) and amino acids and other important nutrients are subsequently incorporated into eggs (Boggs and Gilbert 1979; Boggs and Watt 1981). Due to their substantial investment in the spermatophore, male cabbage whites can also be choosey, paying attention to the same nitrogen-based wing pterins when choosing females (Tigreros et al. 2013, 2014). Given the importance of nitrogen in male and female ornamentation and spermatophore composition, anthropogenic changes in nitrogen availability should have profound impacts on sexual selection dynamics in Pieris butterflies.

We contrasted butterflies from an agricultural and nonagricultural population to investigate how anthropogenic changes in nutrient availability may have impacted sexual selection dynamics. We focused on the largest canola-growing region in the world (north-central United States and central Canada-Figure 1, UN Food and Agriculture Organization 2017). In this region, canola is available as a host plant during most of the cabbage white butterfly's flight period and is subjected to little pesticide use (Kandel et al. 2011; see additional details on this region below). We use a combination of field observations and common garden experiments to test 3 specific predictions. First, we predict that anthropogenic increases in nitrogen result in greater nitrogen availability: host plants in agricultural areas should have higher nitrogen content, as would wild-collected butterflies from those areas. Second, increases in nitrogen availability should select for genetic changes in how nitrogen is assimilated from the diet and allocated to wing ornamentation. More specifically, butterflies from the agricultural population are predicted to allocate more to nitrogen-rich wing pigments, but not necessarily be more efficient at extracting nitrogen from lower quality or novel diets (e.g., a diet-quality-by-population interaction). Third, as nitrogen availability increases, the nutritional benefits to females of re-mating should decrease, resulting in lower re-mating rates (spermatophore counts) and selection for smaller structures that process spermatophores (i.e., fewer "teeth" on the signum). Choosing a specific male may also be less important, resulting in more rapid mating in common garden conditions.

METHODS

Focal populations and overview of approach

We compared an agricultural and nonagricultural population to test how changes in nutrient availability may have impacted sexual selection dynamics. Regions of northern North Dakota (USA) and southern Manitoba and Saskatchewan (Canada) have been a center of canola (*Brassica napus* and *Brassica rapa*) agriculture for over 35 years. The Northeastern region of North Dakota alone plants up to 550 000 acres of canola annually (USDA). *Pieris rapae* in this region feed on canola as a larval host and population densities in late summer are higher than any nearby nonagricultural region.



Figure 1

Map of populations with respect to canola agriculture (in yellow). The hatched region of North Dakota, Manitoba, Saskatchewan, and Alberta represents the largest canola growing region in the world (geographic area modified maps from the USDA and the Canola Council of Canada). Populations were sampled in Northern North Dakota (in and around Langdon, ND) and the Twin Cities (St Paul, MN). Map image from Wikimedia commons (attribution: Lokal_Profil).

Canola is heavily fertilized, with recommended applications ranging from 100 to 250 kg N/ha (Ukrainetz et al. 1975; Kimber and McGregor 1995), with 140-170 kg N/ha suggested in our study region (Franzen 2011). We reasoned that this population has seen a more consistent increase in host plant nitrogen content over the last 30 years than those in our nonagricultural population, which feed on a wider range of host plants in the family Brassicaceae, many of which are found away from commercially fertilized areas. Recent genomic analyses of these populations also suggest that the populations have diverged substantially in nitrogen metabolism (Sikkink et al. 2017), making this a perfect case study to investigate how changing nutrient availability impacts sexual selection dynamics. This broader canola growing region of north-central United States and south-central Canada is the top producing canola region in the world (Figure 1, UN Food and Agriculture Organization 2017), making this a truly unique opportunity to study butterflyhost dynamics in an anthropogenic context. Furthermore, insecticide application rate in this region is low overall and tends to come early in the season when butterfly numbers are low (Kandel et al. 2011). However, as reviewed in more detail in the discussion, there are a range of factors that vary between these populations beside nitrogen input, which emphasizes this as a case study approach.

We used a combination of field- and lab-based approaches to contrast this agricultural population in North Dakota ("ND") with a "control" population in the Twin Cities, Minnesota ("MN"). First, we confirmed that nitrogen content of host plants differed between populations, presumably due in part to differences in fertilizer application. We confirmed this difference in nitrogen availability had impacts on nitrogen limitation of butterflies by also measuring nitrogen content of wild-caught butterflies in both populations. Second, we considered investment in nitrogen-based wing pigmentation (pterins) by measuring wings of wild-collected individuals. To determine whether population differences were due to differences in environmental availability of nitrogen or genetic differentiation between the populations, the populations were reared in common garden conditions. Because we could not exactly replicate canola agriculture in the greenhouse, we used both artificial and plantbased diets that spanned a range of nitrogen concentrations to ensure that patterns were consistent in a range of common garden conditions. We additionally measured the nitrogen content of butterflies raised on these diets to assess their ability to assimilate nitrogen from different diets. Third, we considered dynamics of remating in females. We used spermatophore counts of wild-caught individuals to determine frequency of re-mating in the field. We used controlled cage assays in a greenhouse to measure differences between populations in latency to mate. In addition, we took measures of the bursa copulatrix (which processes spermatophores) as a morphological proxy for the frequency of spermatophore processing; here we used common-garden reared individuals to test for population divergence in this structure. Taken together, these experiments and observations can assess patterns of ornament investment and mating in the field, along with genetic differentiation between the populations with respect to ornamentation and mating behavior.

Nitrogen content of butterflies and plants

We tested variation between populations in host plant leaf nitrogen by collecting leaf samples in June 2014 (plants in North Dakota were sampled 2 weeks later to control for phenology). For the nonagricultural population (MN), we sampled *P. rapae* hosts from 8 different plant genera from gardens, yards, and ditches in the Minneapolis-St Paul area (Supplementary Table 1). In the agricultural population (ND), we collected 3–4 leaves of 5 varieties of canola (*B. napus* and *B. rapa*) from fields near Langdon, North Dakota (where butterflies were sampled). Nitrogen content of dried leaves was quantified at the University of Minnesota Research Analytical Lab using the Dumas method (Matejovic 1995).

We quantified the nitrogen content of whole adult butterflies to estimate their total nitrogen budget. We focused on individuals collected in the field (from multiple field collections in 2012, 2014, 2015, and 2016) and individuals reared in our common garden conditions (specifically the high nitrogen artificial diet and high nitrogen cabbage diets, see below). For comparison groups that spanned multiple years, there were no significant differences in nitrogen content across years (e.g., wild-type MN females, P = 0.42). For this analysis, we pooled butterflies into groups of 3-5 individuals as "biological replicates" with at least 3 biological replicates for each comparison ($\mathcal{N} = 6$ biological replicates for MN females; 3 for MN males; 5 for ND females; 4 for MN males), and at least 2 technical replicates per biological replicate (mean = 2.4 technical replicates). Individual butterflies were dried at 70 °C for at least 24 h. They were weighed as a pooled group, ground with a mortar and pestle into a fine powder and then re-dried at 70 °C before weighing samples for analysis. We used a Costech CN Elemental Analyzer to measure carbon and nitrogen content of butterfly samples using acetanilide as a standard (4 standard replicates, ranging from 0.5 to 4 mg). For each biological replicate, we ran 2 technical replicates (~2 and 4 mg of sample). We measured the mass of each replicate using a microbalance to the nearest 0.0001 mg to factor the precise weight into calculations of total nitrogen content.

Investment in nitrogen-rich wing pigments

In summer 2012, we collected mated, gravid females from the agricultural and nonagricultural populations and brought them back to the lab where eggs were harvested on organic cabbage plants (Brassica oleracea) in $61 \times 61 \times 61$ cm mesh cages. We conducted 2 common garden experiments, rearing populations in the lab on artificial diet and in the greenhouse on potted cabbage plants. For each diet type, we additionally varied the nutrient content through fertilizer manipulations or altering artificial diet ingredients (see below). We were primarily interested in rearing the 2 populations in a range of common garden nutritional conditions to test for genetic differences in allocation to nitrogen-based wing ornamentation. Thus, we created a range of diets, both artificial and plantbased, that varied in nutrition. We primarily manipulated nitrogen, but also simultaneously manipulated potassium, phosphorus, and to some extent sodium, in part due to the methods for diet manipulation (see below), and in part because all of these nutrients are also enriched in agricultural areas (e.g., with N-P-K fertilizer addition and sodium accumulation with any irrigation; Snell-Rood et al. 2015). We additionally collected wild males in 2012 to compare body size and wing pterin investment in wild-collected males.

Common garden rearing on artificial diet

Larvae from each population were transferred with paintbrushes to either a low or high nitrogen artificial diet treatment (in a fully factorial manner) within 7–10 days of the eggs being laid (late first or early second instar). Diets were constructed based on an initial pilot experiment. The 2 diets used in the present experiment differed by the amounts of cellulose, casein, and torula yeast: high nitrogen diets included 10 g cellulose, 30 g casein, and 12 g torula yeast while low nitrogen diets included 24 g cellulose, 20 g casein, and 8 g torula yeast. Both treatments were composed of 50 g wheat germ, 15 g cabbage flour, 24 g sucrose, 9 g of Wesson salt mix, 3.6 g cholesterol, 10.5 g vitamin mix, 0.75 g methyl paraben, 1.5 g sorbic acid, 3 g ascorbic acid, 0.175 g streptomycin, and 5 mL linseed oil per 800 mL water. Both treatments were mixed with 15 g agar and poured into 4-ounce cups, stored at 4 °C for up to 3 weeks. Larvae were reared on artificial diet in groups of 3 per 4-ounce cup in climate chambers on a 14:10 light:dark photoperiod at 23 °C. This density minimizes disease and allows ad libitum food availability. Adults were removed within 24 h of emergence and stored in glassine envelopes in a sealed container at -20 °C until measurements were made.

To measure nutrient content of the artificial diet, diet samples were taken from 3 diet cups from each diet treatment. Nitrogen content was quantified at the University of Minnesota Research Analytical Lab using the Dumas method (Matejovic 1995). ICP-AES was used to measure potassium, phosphorus, and sodium. Overall, the 2 artificial diets had comparable nitrogen content to host plants collected from the nonagricultural (MN) population. The high nitrogen artificial diet had 25% more nitrogen than the low nitrogen diet (Supplementary Table 2). Because nitrogen was manipulated in part through yeast content, the high nitrogen diet also had 14% more potassium, 17% more phosphorus, and 16% more sodium (Supplementary Table 2).

Common garden rearing on host plants

We additionally reared larvae from both populations on host plants grown in the greenhouse, in a fully factorial design. Cabbage plants (*B. oleracea* var. Copenhagen Market), were planted in a mixture of soil, vermiculite, and sand (50% soil, 25% vermiculite, 25% sand). Before the start of feeding experiments, high and low nitrogen treatments were initially fertilized with 5 and 0 g of fertilizer, respectively (14-14-14 Osmocote slow release NPK fertilizer); 6 weeks later, high and low nitrogen treatments were fertilized with 3 and 1 g of fertilizer, respectively. Following the conclusion of the experiment (8 weeks after the start), new growth and old growth tissues were sampled from plants from each treatment for nitrogen analyses (larvae were no longer feeding on these plants); leaves from a single plant were pooled for analysis because larvae tended to completely defoliate plants before moving to adjacent plants (see below).

Field-collected gravid females laid eggs directly on the high and low fertilized plants and eggs were left to develop in mesh sleeve cages within the greenhouse. Thus, all offspring in this experiment came directly from field-collected mothers. Temperatures fluctuated daily in the greenhouse (generally between 24 °C and 30 °C), so analyses of development time were not performed on these individuals. As individual plants were consumed, additional plants of the same treatment were added to the cage and caterpillars were transferred manually or were allowed to crawl to new plant material. Caterpillars pupated within the mesh cage; pupae were removed for emergence in the lab, where they were sacrificed within 24 h of emergence. Adults were stored in glassine envelopes in a sealed container at -20 °C until measurements were made.

Element content of host plants was measured as for the artificial diet. Greenhouse grown cabbage used in this experiment had 59% higher nitrogen in the fertilized treatment. Because nitrogen was manipulated through a general fertilizer application, the high nitrogen diet also had 29% higher phosphorus, but no difference in potassium (Supplementary Table 2). Our relatively low levels of fertilizer application resulted in the greenhouse-raised cabbage having significantly lower nitrogen content than all wild-collected host plants (Supplementary Table 2), although it is possible that this also reflects sampling of the plants well after fertilizer application and the time course of the experiment.

Pterin measurements

To measure wing pterin content we used an Ocean Optics, Inc. Jaz Spectrophotometer. The reflectance wand was held at a fixed angle and 3 replicate measurements were taken per individual by placing the wand flush in the middle of the central wing cell (wings were removed from individuals). To determine pterin content in the wings, we calculated 3 validated proxies of wing pterin concentration (Wijnen et al. 2007) using methods adopted from Morehouse and Rutowski (2010). First, average reflectance was calculated between wavelengths 300-375 and 450-550 nm. The mean of these 2 average wavelength ranges was calculated to obtain the "midpoint" (R₅₀), and the corresponding wavelength at the midpoint was determined ("lambda" or midpoint wavelength, λ_{50}). Finally, the slope tangent to the midpoint was calculated ("beta" or β_{50}). Across all specimens, pterin measures were all positively correlated with each other (λ_{50} -R₅₀: $\rho = 0.48$, P < 0.0001; β_{50} -R₅₀: $\rho = 0.69, P < 0.0001; \lambda_{50} - \beta_{50}; \rho = 0.11, P = 0.02)$. However, given the low correlations between 2 of the measures, we opted to treat each metric separately in analyses. To control for effects of wing wear and scale loss on pterin measurements, all common garden reared individuals for spectrophotometer analyses were sacrificed within 24 h of emergence. We additionally measured forewing area as a measure of body size. Wings were removed from butterflies and placed flat for imaging with a Canon Rebel T3 fitted with a 50 mm macro lens; forewing area was measured in Image J (NIH).

Mating dynamics

In summer 2014, we again sampled our 2 focal populations for measurements of mating frequency and a set of common garden experiments to investigate mating behavior.

Spermatophore counts

We used spermatophore counts to measure the number of times a given female mated. Females were sampled in the agricultural population (ND) in 2014 (N = 25), and the nonagricultural (MN) population in 2014 (N = 14), 2015 (N = 23), and 2016 (N = 12). While we sampled the North Dakota population only once for this comparison (N = 25 dissected females), we sampled the population during peak population numbers (18–20 August 2014) over 3 days, at 5+ locations spread over a 400 mi² area. To count spermatophores, abdomens were dissected in 1×PBS with a Leica M165C stereomicroscope at 10–20×. The bursa copulatrix was removed and opened with fine forceps, and individual spermatophores identified by one observer (ESR).

Common garden measurement of mating frequency and signum characteristics

Females collected from the 2 focal populations in August 2014 were raised through 2 generations on greenhouse plants (potted and fertilized cabbage and radish—see Sikkink et al. 2017). More specifically, the first generation was allowed to mate in large groups (N > 20 males and females from each population per cage in 2 replicate cages) before rearing of the second generation on potted *B. rapa* on a short (10 h) photoperiod to induce diapause. Diapaused pupae

were stored in the dark at 6 °C until May 2015 when diapause was broken with exposure to a 24 h photoperiod. For the mating assay, males (in groups of 8) were housed in $61 \times 61 \times 61$ cm mesh cages in the greenhouse for 2 days before introduction of virgin females (8 per cage). Each mating cage contained males from a given population and females from either the same or opposite population in fully factorial combinations with 3 replicates of each combination (12 cages total). Butterflies were given 48 h to mate, before being frozen and stored at -20 °C before dissection to count spermatophores and measure the bursa copulatrix (see below). During the 48 h mating period, butterflies had access ad libitum to 10% honey solution (on yellow sponges); cages also contained potted nonhost plants for extra humidity and habitat structure. Abdomens of females were dissected in 1× PBS on a Leica M165C stereo microscope. The bursa copulatrix was removed and spermatophores counted by one observer (TP). Additionally, the signum of the bursa copulatrix, the toothed structure that bursts open the spermatophore, was placed flat and imaged. The number of teeth on each lobe of the signum was counted under the microscope and the area and perimeter of each lobe was measured in Image J (NIH). For this analysis, the body size of each individual was measured as forewing area as described above. As body size did not vary with signum tooth density, it was not included in final analyses.

Data analysis

JMP 13.0 (SAS Institute) was used for statistical analyses. All measurements are available in DRYAD (doi:10.5061/dryad.v0cm4k9). For measurements of plant nitrogen content, we used ANOVAs to test for effects of population origin, separating analyses for all Brassicaceae collected and a subset of mustards listed by Scott (1992) as preferred larval hosts. For measures of butterfly nitrogen body content, we included at least 2 technical replicates per biological replicate. Thus, we used mixed-effects models testing for fixed effects of population and sex, including biological replicate as a random effect; these analyses were run separately for wild-collected versus lab-reared butterflies. For mixed effects models, we report "error degrees of freedom" as the Kenward–Roger first-order approximation ("DfDen" in JMP fixed effect test output).

For measures of wing pterin investment, we used at least 3 replicate wing spectrophotometer measures for each individual. We used mix-effects models that tested for fixed effects of population and sex, treating individual as a random effect. Analyses were run separately for each diet type (cabbage-reared, artificial diet, and wild-collected) and each of the 3 pterin measures described above. ANOVAs were used to test for effects of population on body size (forewing length), development time, and signum tooth density, controlling for sex where appropriate. Finally, we used chi-squared tests to test for differences between populations in spermatophore counts and probability of mating in a common garden test. We report results of all analyses; model selection was not performed.

RESULTS

Nitrogen availability in agricultural populations

Host plant foliar nitrogen was greater in the agricultural (North Dakota) population of *P. rapae*. Samples of host plants from 6 agricultural fields (and one adjacent ditch, Supplementary Table 1) revealed significantly higher nitrogen content of field-collected canola (6.17%) relative to a sampling of *P. rapae* preferred and commonly encountered hosts from the nonagricultural (Minnesota)

population from 6 different locations (e.g., garden, ditches, yards; Figure 2A: 4.78%, $F_{1,17} = 7.5$, P = 0.01). A broader pool of all Brassicaceae that included less preferred, but potential hosts (Scott 1992) in the nonagricultural population (MN), showed an even greater difference between populations ($F_{1,24} = 8.74$, P = 0.007).

Wild-collected butterflies from the agricultural population (ND) had higher total body nitrogen content relative to the nonagricultural population (Figure 2B; GLMM: population: $F_{1,15} = 13.8$, P = 0.002, sex: $F_{1.15} = 1.56$, P = 0.23, N = 44 observations, 18 biological replicates). Males collected from the agricultural population (ND) had significantly greater wing pterins for one (R_{50}) of the 3 measures of pterin investment (N = 93 observations, 31 individuals; R_{50} : $F_{1,29}$ = 4.89, P = 0.035, LSM MN: 49.4; ND: 51.3), but not the other 2 (λ_{50} : $F_{1,29} = 1.18$, P = 0.29, LSM, MN: 427.4, ND: 429.3; β_{50} : $F_{1,29} = 0.47$, P = 0.50, LSM MN: 2.36, ND: 2.15). Wild-collected females from the agricultural population (ND) were significantly larger than those from the nonagricultural population (MN; forewing area: $F_{1.55} = 6.89$, P = 0.01, mean [SE] cm²: MN: 1.99 [0.03]; ND: 2.11 [0.03]); however, this pattern was reversed in males (forewing area: $F_{1.43} = 71.1$, P < 0.0001, mean [SE] cm²: MN: 2.21 [0.16]; ND: 1.73 [0.21]).

Nitrogen assimilation and allocation to ornamentation in common garden

To determine whether population differences in nitrogen assimilation and allocation were a result of genetic or environmental differences, we reared butterflies from both populations in 2 common garden experiments—in the lab on an artificial diet and in the greenhouse on potted cabbages (*B. oleracea*, the same genus as canola). The nitrogen content of each diet type was further manipulated to assess the response of each population to a range of nutritional conditions. Regardless of rearing diet or sex, the agricultural population (ND) allocated significantly more to nitrogenbased wing ornamentation, as indicated by 2 of our 3 measures of pterin investment (λ_{50} and R_{50} ; Table 1, Figure 3, Supplementary Figure 1). Pterin investment also tended to be higher when reared on cabbage (vs. the artificial diet) and in males versus females (Table 1, Supplementary Figure 1). There were significant interactions between diet and population (Table 1), such that differences



Figure 2

Variation between populations in nitrogen content of (A) host plants and (B) butterflies. Host plant material and wild-collected butterflies sampled from the agricultural population (ND; green) had significantly greater nitrogen content than those collected from the nonagricultural population (MN; purple). However, butterflies from the agricultural population reared in a common garden had comparable or lower total body nitrogen. Error bars indicate standard error.

between populations tended to be greater on the higher nitrogen diets (λ_{50}) or on the cabbage relative to the artificial diet $(R_{50};$ Figure 3, Supplementary Figure 1, following Tukey HSD correction of mean comparisons for each interaction).

While individuals from the agricultural population (ND) had relatively smaller forewing area on the artificial diet, they had significantly larger wings on the cabbage diet (Table 1, Supplementary Figure 2). The agricultural population developed significantly faster on artificial diet (LSM ND: 31.4 days), relative to the nonagricultural population (MN: 34.0 days; N = 112 individuals, population: $F_{1,108} = 15.5$, P = 0.0001, Diet: $F_{1,108} = 0.49$, P = 0.48, sex: $F_{1,108} = 0.05$, P = 0.81). The nitrogen content of individual butterflies did not vary with population origin when reared on cabbage (Figure 2; GLMM: population: $F_{1,10} = 0.56$, P = 0.47, sex: $F_{1,10} = 4.98$, P = 0.049, M > F, N = 26 observations, 13 biological replicates), however, when reared on artificial diet, the agricultural

population (ND) had significantly lower total body nitrogen content, suggesting they are less efficient at assimilating nitrogen from their diet relative to the nonagricultural population (Figure 2; GLMM: population: $F_{1,5} = 8.23$, P = 0.035, sex: $F_{1,5} = 0.17$, P = 0.69, N = 20 observations, 8 biological replicates).

Mating dynamics in the field and common garden

In the wild, females from the agricultural population (ND) were significantly less likely to mate with multiple males (Figure 4, $\chi^2 = 19.49$, P = 0.0002, N = 74 females, with 25 from ND)—most wild-collected females had only 1 spermatophore, compared with 1.6 spermatophores on average in the nonagricultural population (MN). This difference remained significant when the comparison was restricted to 2014 (the time span for which ND females were sampled: $\chi^2 = 21.89$, P < 0.0001, N = 39 females). In common

Table 1

Population effects on wing pterin investment and body size in a common garden

| Factor | λ_{50} | β_{50} | R_{50} | Forewing area |
|------------------------------------|--------------------------------|---------------------------------|---------------------------------|---------------------------------|
| Sex | $F_{1,101} = 55.9, P < 0.0001$ | $F_{1.101} = 112.8, P < 0.0001$ | $F_{1,101} = 190.6, P < 0.0001$ | $F_{1,100} = 3.15, P = 0.08$ |
| \mathcal{N} content ^a | $F_{1,101} = 7.03, P = 0.001$ | $F_{1,101} = 4.72, P = 0.01$ | $F_{1.101} = 0.34, P = 0.72$ | $F_{1,100} = 2.05, P = 0.13$ |
| Diet type | $F_{1,101} = 48.5, P < 0.0001$ | $F_{1,101} = 0.09, P = 0.76$ | $F_{1.101} = 3.16, P = 0.08$ | $F_{1,100} = 156.4, P < 0.0001$ |
| Population | $F_{1,101} = 12.0, P = 0.0008$ | $F_{1,101} = 2.23, P = 0.13$ | $F_{1.101} = 20.5, P < 0.0001$ | $F_{1,100} = 0.44, P = 0.51$ |
| Population × diet | $F_{2,101} = 1.44, P = 0.23$ | $F_{2,101} = 0.06, P = 0.80$ | $F_{2,101} = 10.5, P = 0.002$ | $F_{2,100} = 16.5, P < 0.0001$ |
| Population $\times \mathcal{N}$ | $F_{2,101} = 6.31, P = 0.003$ | $F_{2,101} = 1.20, P = 0.31$ | $F_{2,101} = 0.25, P = 0.78$ | $F_{2,100} = 4.07, P = 0.02$ |

Individuals were reared on either artificial diet or potted cabbage ("diet type"); diet nitrogen content was additionally varied within each diet type ("N content"). For wing coloration measurements, the table shows results from a mixed effects model that treated individual as a random effect (N = 330 observations total, 109 individuals).

^aNested within diet type.



Figure 3

Effects of population and diet on 2 measures of wing pterin investment. Shown are results from mixed effects models where individual is treated as a random effect (330 total observations on 109 individuals), testing for an overall population effect (statistics reported in Table 1). For visualization purposes, results are plotted separately for each sex and the cabbage diet only, but all diets and pterin measurements are shown in Supplementary Figure 1. Green lines indicate the agricultural (ND) population while purple lines indicate the nonagricultural (MN) population. Across both sexes and diet types, the agricultural population allocated significantly more to wing pterins for these 2 pterin measurements (no significant population effect for the third pterin measurement). Error bars indicate standard error and means represent least square means from a general linear mixed model that controls for diet and technical replicates.



Figure 4

Patterns of mating in wild populations. (A) Spermatophore counts of females collected in the wild differed significantly between nonagricultural (MN, purple) and the agricultural (ND, green) populations. Females from the agricultural population were significantly more likely to mate only once relative to females from the nonagricultural population. (B) Females from the agricultural population (ND, green) were more likely to have mated after 48 h in common garden conditions relative to the nonagricultural population (MN, purple).



Figure 5

Signum characteristics vary between populations. Each population was reared for 2 generations in the lab (on plants) before dissection of the bursa copulatrix. The nonagricultural population (MN, purple) had a significantly greater density of teeth on the signum than the agricultural population (ND, green). Pictured are representative individuals (with the average signum tooth density for that population). Error bars indicate standard error.

garden mating conditions, however, virgin females from the agricultural population (ND) were significantly more likely to have mated after 48 h than females from the nonagricultural population (Figure 4; $\chi^2 = 6.85$, P = 0.0089, $\mathcal{N} = 63$ females), regardless of the population of origin of the males with which they were paired ($\chi^2 = 0.043$, P = 0.84, $\mathcal{N} = 63$ females).

The 2 populations also showed differences in the morphology of the signum of the bursa copulatrix—the structure in female butterflies that breaks open spermatophores for digestion. After rearing the populations for 2 generations in the lab (on plants), females descended from the nonagricultural population (MN) had a significantly greater density of teeth on the signum (Figure 5, $F_{1,61} = 30.77$, P < 0.0001, $\mathcal{N} = 63$ females).

DISCUSSION

We used a case study of an agricultural population of cabbage white butterflies to test the hypothesis that anthropogenic increases in nutrient availability should alter sexual selection dynamics. Overall, our results are consistent with the idea that increased nutrient availability results in the evolution of increased ornamentation (but not necessarily correlated with male quality) and a reduction in the benefits to females of polyandry. Host plant nitrogen content is significantly higher in the agricultural population (Figure 2), consistent with the high fertilizer application in the area. Correspondingly, wild-collected butterflies from the agricultural population also have higher whole-body nitrogen content relative to the nonagricultural population (Figure 2); similarly, wild-collected females from this population are larger. Taken together, this suggests that individuals in the agricultural population, especially females, have more available nitrogen in their diets and are thus less nitrogen-limited.

Analyses of mating dynamics indicate that the increased availability of nitrogen has led to a decline in female re-mating in the agricultural population. Spermatophore counts of wild-collected individuals suggest that females tend to mate only once in the agricultural population, while females in the nonagricultural population mate on average 1.6 times (Figure 4). Furthermore, the signum, the structure of the bursa copulatrix that bursts open spermatophores within the female (Tschudirein and Benz 1990; Galicia et al. 2008), has a significantly higher tooth density in females from the nonagricultural population (Figure 5), which aids in breaking open and processing spermatophores (Tschudirein and Benz 1990; Galicia et al. 2008). Because females from the nonagricultural population have less available nitrogen, they may experience a greater benefit from increasing throughput of spermatophores (Kaitala and Wiklund 1994; Karlsson 1998). In contrast, females in agricultural areas may benefit less from re-mating as more nitrogen is available from their larval diet, reducing their need for adult protein sources (Mevi-Schutz et al. 2003). Amino acid composition of fertilized canola nectar may also be higher (Gardener and Gillman 2001; Gijbels et al. 2015) which should additionally lead to a decline in female re-mating as adult diet improves (Boggs 1990). Further experiments could confirm that increased nitrogen availability in the agricultural population indeed has beneficial effects on fecundity and offspring quality, to exclude the alternate hypothesis that novel increases in pterin investment could be unattractive.

These data suggest that as nutrient availability increases, re-mating decreases and females are more likely to mate singly. However, females from the agricultural population are not necessarily choosier. Common garden mating experiments showed that virgin female cabbage whites from the agricultural population were more likely to have mated after 48 h, relative to the nonagricultural population, regardless of the population origin of males they were paired with (Figure 4). Assuming that latency to mate is correlated with choosiness (e.g., Berglund 1994; Bateman et al. 2001), individuals from the agricultural population appear to be *less* choosy than those from the nonagricultural population, but further behavioral trials are necessary to solidify this interpretation. Patterns of female choice could additionally be interacting with male choice of females based on their pterin investment (Tigreros et al. 2014), which is also higher in the agricultural population (Figure 3). Indeed, it's possible that higher female-female variance in re-mating in the nonagricultural population could result from greater differences in the ability of females to attract mates based on higher variation in larval diet quality (Bergstrom and Wiklund 2002; Tigreros et al. 2013).

As nutrient availability increases, we also predicted that the utility of sexual traits as indicators of quality would lessen. Two of our results together support this idea. First, both males and females from the agricultural population, when reared in common garden, allocated relatively more to nitrogen-rich wing pterin ornamentation (Figure 3). This makes sense given that nitrogen availability is consistently higher in the agricultural populations (Figure 2): there should be a benefit to an evolutionary shift in how nitrogen is allocated to this ornamentation given that pterins are important for both females choosing males (Morehouse and Rutowski 2010) and males choosing females (Obara et al. 2008; Tigreros et al. 2014). However, this shift in ornament allocation does not correspond to genetic differences in quality, at least in terms of the ability to assimilate nitrogen from the diet. When reared on plants in a common garden, the agricultural population does not differ in body nitrogen content; however, on artificial diet, they have significantly less body nitrogen content than the nonagricultural population (Figure 2). The agricultural population appears to be less efficient at assimilating nitrogen from a range of diets, consistent with the fact that nitrogen metabolism genes have diverged significantly between populations (Sikkink et al. 2017). While patterns of pterin investment may not currently be an indicator of genetic quality (in terms of nitrogen assimilation ability), utility of coloration as an indicator may be restored over time as the traits are further elaborated (Kokko et al. 2002; Prum 2010) or as females attend to a different trait in mate choice.

Several additional observations suggest that the 2 populations have diverged in significant ways with respect to nitrogen assimilation from different diets. To measure responses to a range of diets, we chose to contrast populations on 2 diet types (artificial and cabbage) with variation in nutritional quality within each type. Overall, the agricultural population did relatively worse on the artificial diet, both in terms of overall body size (Supplementary Figure 2) and the amount of total nitrogen assimilated (Figure 2). When reared on plants, the agricultural population tended to be larger and assimilate more nitrogen (Supplementary Figure 2, Figure 2). Given that the nitrogen content of the artificial diet was actually higher than the plants in this common garden experiment (Supplementary Table 2), this suggests that overall differences between the populations in response to diet type may not derive solely from differences in nitrogen availability. It is possible that differences in diet breadth could be explaining some of the differences between responses to plant and artificial diets. The agricultural population feeds mostly on canola (B. napus and B. rapa), while the nonagricultural population feeds on 20+ species from a wider variety of genera (e.g., Brassica, Raphanus, Barbarea, Lepidium, Scott 1992). It is likely that the broader niche breadth of the nonagricultural population has contributed to their ability to assimilate nitrogen more efficiently from a relatively novel, artificial diet while the agricultural population is more limited in its ability to extract nitrogen efficiently from a range of diets. It is also possible that other nutritional differences between populations could be driving the responses to different diets. As nitrogen availability increases, other nutrients may become relatively more limiting and cause evolutionary changes in nutritional physiology; given that phosphorus and potassium are also applied in crop fertilizer, it is possible that micronutrients such as magnesium or calcium may be limiting in the agricultural population, or, from a macromolecular perspective, lipids or essential fatty acids (Friend 1958; Fraenke 1959; Vanderzant 1965).

Interestingly, wild-collected males from the agricultural population are relatively smaller. This is consistent with observations from other pierid butterflies where selection on male body size (and resulting sexual size dimorphism) increases with polyandry due to selection on male ejaculate production, which increases with body size (Wiklund and Forsberg 1991; Wiklund and Kaitala 1995). Decreases in female polyandry in the agricultural population may have caused relaxed selection on male ejaculate production and thus body size. This could also explain the observation from our common garden experiments that revealed that males from the agricultural population develop significantly faster. Despite their smaller body size, the higher nitrogen content of diets of males in the agricultural population suggests that these males could still be producing high quality spermatophores (Ferkau and Fischer 2006), especially if individual males are not mating as frequently (Kaitala and Wiklund 1994; Bissoondath and Wiklund 1996), although this supposition would need to be confirmed with chemical analyses of spermatophores dissected from freshly mated females.

The agricultural population studied here is truly unique-it is easily the largest agricultural population of cabbage whites in North America that experiences agricultural host plants during their entire flight period (other Brassicaceae crops are limited in their planting season to early in the year in places like California). While this presents an exciting opportunity to study extreme anthropogenic environments, it is a case study that is limited in its ability to isolate one independent variable. In addition to using higher nitrogen host plants, the agricultural population also has a narrower diet breadth, a higher population density, a shorter breeding season, and higher average wind speeds. The agricultural population in northern North Dakota also experiences periodic exposure to insecticides early in year, approximately 25% of the time for control of flea beetles (Kandel et al. 2011). However, insecticide application in Canadian canola is recommended for a broader range of pests (Canola Council of Canada 2017) and recently has seen an increase in use and ecosystem contamination of neonicotinoids (Main et al. 2014), suggesting that this broader agricultural population is likely experiencing stronger insecticide exposure which may have negative fitness consequences on butterflies (Sinha et al. 1990; Pecenka and Lundgren 2015; Whitehorn et al. 2018). It is difficult to determine whether nitrogen or one of these other environmental variables is driving the changes in ornament investment and re-mating dynamics. In particular, the increases in nitrogen and host plant availability have resulted in a large increase in population density in the agricultural population, which is known to impact sexual selection dynamics. However, empirical studies from insects, fish, and mammals suggest that increases in density tend to decrease the intensity of sexual selection in males as more males gain access to females and variance in male mating success declines (Clutton-Brock et al. 1997; Conner 1989; McLain 1992; Jirotkul

1999), although there is variation across systems in how population density affects sexual selection dynamics, especially in mating systems that exhibit territoriality (Fleming and Gross 1994; Moller and Ninni 1998) or nuptial gifts (Gwynne 1984). For instance, giftgiving crickets shift from male-male competition to female-female competition (and higher female-female mating variance) as density increases (Gwynne 1984). Regardless, these predictions based on density run counter to our observation that ornamentation increases in the agricultural population in both males and females, which is more consistent with a nutritional explanation. Given the limitations of a case study approach, an experimental evolution approach, or a broader population comparison that focused on just one variable, such as nitrogen deposition, fertilizer application, or population density, might allow clarification of the primary causal factors behind the factors observed here. While the present population comparison is limited in its ability to disentangle the individual variables driving the evolution of these sexual traits, it also presents a unique and powerful opportunity to study the complexities of sexual selection in a rapidly changing environment.

The field of sexual selection has long been interested in condition-dependence of ornaments (Iwasa and Pomiankowski 1991; Andersson 1994; Rowe and Houle 1996). More recently, it has been suggested that a more nutritionally explicit consideration of "condition" can yield novel insights in the ecology and evolution of sexual traits and reproduction (Morehouse et al. 2010; Simpson and Raubenheimer 2011). Indeed, considering precise nutrient requirements has already been applied to the expression of sexual traits in a range of systems (Bertram et al. 2006; Sentinella et al. 2013; Harrison et al. 2014), including with respect to nutrients changing due to human activity (Goos et al. 2014). We argue that considering nutrition from a stoichiometric or elemental perspective (Sterner and Elser 2002) also allows links between ecosystem nutrient cycling and understanding the development and evolution of sexual traits in the Anthropocene (Snell-Rood et al. 2015). More broadly, an understanding of ecological stoichiometry and changes in nutrient cycling is beginning to yield insights on how humans are affecting the evolution of a broad suite of traits, from sexual ornaments to genome content and growth rates (Acquisti et al. 2009; Frisch et al. 2014).

This work introduces a novel mechanism by which humans may be impacting mating systems. A large body of research has highlighted how humans may affect the efficacy of signaling systems by altering signal transmission. For instance, increasing phosphorus affects eutrophication and turbidity of aquatic systems, which can negatively affect mate choice in a variety of fish species (Seehausen et al. 1997; Jarvenpaa and Lindstrom 2004). In birds, anthropogenic noise can affect the efficacy of acoustic signals (Halfwerk et al. 2011; des Aunay et al. 2014). The present research builds on work linking anthropogenic environments and sexual selection by suggesting that rapid and extreme changes in nutrient availability can alter-and likely relax-sexual selection intensity. As nitrogen becomes more available, nutritional benefits of re-mating are reduced and female re-mating rates decline. At the same time, females appear to be less choosy, mating quickly after emergence. Finally, both sexes allocate more to ornamentation, even if it is not indicating the ability to assimilate nitrogen from the diet. Studying divergence in sexual traits and mating behavior in anthropogenic environments offers an opportunity to investigate aspects of sexual selection in real time (Snell-Rood et al. 2015), clarifying questions that are challenging to address for signals and preferences that may have arisen thousands of generations ago.

SUPPLEMENTARY MATERIAL

Supplementary tables and figures are available at *Behavioral Ecology* online.

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Data Archival Location: Analyses reported in this article can be reproduced using the data provided by Espeset et al. (2019).

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