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# Nitrogen fixation in the stag beetle, *Ceruchus piceus* (Coleoptera: Lucanidae): could insects contribute more to ecosystem nitrogen budgets than previously thought?

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Nitrogen (N) is a key nutrient required by all living organisms for growth and development, but is a limiting resource for many organisms. Organisms that feed on material with low N content, such as wood, might be particularly prone to N limitation. In this study, we investigated the degree to which the xylophagous larvae of the stag beetle *Ceruchus piceus* (Weber) use associations with N-fixing bacteria to acquire N. We paired acetylene reduction assays by cavity ring-down absorption spectroscopy (ARACAS) with $^{15}N_2$ incubations to characterize rates of N fixation within *C. piceus*. Not only did we detect significant N fixation activity within *C. piceus* larvae, but we calculated a rate that was substantially higher than most previous reports for N fixation in insects. While taking these measurements, we discovered that N fixation within *C. piceus* can decline rapidly in a lab setting. Consequently, our results demonstrate that previous studies, which commonly keep insects in the lab for long periods of time prior to and during measurement, may have systematically under-reported rates of N fixation in insects. This suggests that within-insect N fixation may contribute more to insect nutrition and ecosystem-scale N budgets than previously thought.

*Key words:* nitrogen, fixation, xylophagous, beetle, larva

## Introduction

Nitrogen (N) is one of the fundamental building blocks of life, providing the basis for protein synthesis and other essential functions in all organisms. Although it is one of the commonest elements found on Earth, comprising >78% of the atmosphere as dinitrogen gas, many of Earth's ecosystems are N-limited (Elser et al. 2007, LeBauer and Treseder 2008) because dinitrogen is chemically inaccessible to most organisms. N limitation is a particular challenge for organisms that consume the woody xylem tissue of plants, which has the lowest N content of any living tissue, averaging 0.2% N by mass (Mattson 1980). Organisms feeding primarily on wood are classified as xylophagous, a term derived from the Ancient Greek *xylon phagein*, literally translated as "to eat wood."

Among the wide array of xylophagous organisms, xylophagous beetles are a particularly diverse group, and are known to play an important role in regulating the processes of decomposition and nutrient cycling in temperate forested ecosystems (Ausmus 1977, Stokland et al. 2012). Decomposition is the primary pathway by which nutrients in the woody tissues of plants are returned to the soil and made available to living plants, and among animals xylophagous beetles may be second only to termites in their consumption of dead wood (Ulyshen 2016). The activities of xylophagous beetles increase the rate of decomposition of dead wood directly through their comminution of wood fragments (Leach et al. 1937, Edmonds and Eglitis 1989, Schowalter and Zhong 1989, Müller et al. 2002, Angers et al. 2012) and indirectly through a variety of mechanisms including production of frass, inoculation of wood with microbes, and creation of spatial heterogeneity in wood (Ausmus 1977, Ulyshen 2016). Many xylophagous beetle species are under threat in temperate ecosystems (Dahlberg and Stokland 2004), where they are understudied in comparison to the tropics, even though the contribution of xylophagous beetles to ecosystem function may be more significant in temperate zones (Ulyshen 2016).

The stoichiometric N requirements of xylophagous beetles greatly outweigh N availability in their diet (Cowling and Merrill 1966), and it is not fully understood how they overcome the extremely high C:N ratio in decomposing wood to extract adequate N resources. One strategy used by many species of invertebrates is to house microbial symbionts that recycle inaccessible N from insect waste products and use the recycled N to synthesize N-rich compounds that are made available to their insect host (Nardi et al. 2002, Douglas 2009). Another strategy employed by many xylophagous beetles is to obtain N by consuming the fungi associated with decaying wood (Birkemoe et al. 2018), which use foraging mycelia to access N external to the woody substrate (Boddy and Watkinson 1995). A third strategy used by some species of xylophagous beetle—the focus of the present study—is to form associations with N-fixing bacteria, which are housed in the beetle's gut, to supplement their dietary intake (Bar-Shmuel et al. 2020).

N fixation is the process by which atmospheric $N_2$ is converted by specialized bacteria into biologically available ammonium ($NH_4^+$). A variety of plant species form mutualistic symbioses with N-fixing bacteria, in which the bacteria provide nitrogen to their host in exchange for carbon (Vitousek et al. 2002). Potential N-fixing bacterial species have been isolated from the hindguts of many invertebrate species (summarized in detail in Bar-Shmuel et al. (2020)), although the precise nature of the exchange between bacteria and host is not fully understood. N fixation has been relatively well-studied in termites (e.g., Breznak et al. 1973, Reid and Lloyd-Jones 2009, Meuti et al. 2011), and has also been directly measured in several beetle species, including *Dorcus rectus* (Kuranouchi et al. 2006), *Dendroctonus valens* (Morales-Jiménez et al. 2009), and *Anoplophora glabripennis* (Ayayee et al. 2014). Given the number of invertebrate species that feed primarily on extremely N-poor diets, and the number of potential associations between insects and N-fixing bacteria that have already been identified, it seems likely that N fixation may be a much more commonly employed strategy of nutrient acquisition than is currently known (Bar-Shmuel et al. 2020).

In this study, we investigated the rates of N fixation in larvae of the stag beetle *Ceruchus piceus* (Weber) in a temperate forest in New York State, USA. *C. piceus* is a member of the Lucanidae, and is commonly found in downed deciduous trees in the later stages of decay (Ratcliffe 2002). Female *C. piceus* deposit eggs on or under the bark of suitable logs; the newly-hatched larvae burrow into the wood where they remain, feeding on the decaying xylem tissue, until maturing into adults (Percy et al. 2000). Given that N fixation has not been studied in this species before, we first asked: *Does N fixation occur at detectable rates in* C. piceus *larvae?* Upon detecting N fixation activity, our next question was: *What is the distribution of rates of N fixation within* C. piceus *larvae?* While answering this question, we discovered that the rate of N fixation changed rapidly after larvae were removed from their natural habitat. This discovery led us to ask an additional question: *How quickly does N fixation within* C. piceus *larvae change after they are removed from their natural habitat?*

## Methods

### Study Site and Specimen Collection

We collected the data for this study between July and October 2020 in Black Rock Forest, a 1550-ha forest preserve located in the Hudson Highlands Region of south-eastern New York State, USA (41.420°N, 74.009°W). Mean annual precipitation is around 1.2 m, and mean monthly temperatures range from approximately –2.7 °C in January to 23.4 °C in July (Schuster et al. 2008). The site is rocky and generally nutrient-poor, with elevation ranging from 110 to 450 m above sea level. The forest canopy is dominated by red oak (*Quercus rubra*) and chestnut oak (*Quercus montana*), but sugar maple (*Acer saccharum*), black birch (*Betula lenta*), and yellow birch (*Betula alleghaniensis*) are also common species (Schuster et al. 2008).

We collected *Ceruchus piceus* larvae from approximately 12 different dead, downed logs in the later stages of decay in different areas of Black Rock Forest. Due to the advanced stage of decay, it was not possible to accurately identify the tree species that the wood was collected from. We brought large pieces of wood (approximately 15–60 cm in length, 10–20 cm in diameter) that we had identified in the field as suitable habitat for *C. piceus* into the laboratory, and extracted larvae immediately prior to being analyzed. We usually collected the wood in the morning (~9–11 am), and extracted most of the larvae from the wood within 6 hours of being brought into the laboratory, although we did keep some pieces of wood in the laboratory overnight and extracted the larvae the following day (within 30 h). Given the size of the wood pieces, we expect that the internal temperature and moisture levels of the wood did not change substantially over time.

### Nitrogenase Assays and Respiration Measurements

We measured nitrogenase activity in *C. piceus* larvae ($N$ = 37) using Acetylene Reduction Assays by Cavity ring-down Absorption Spectroscopy (ARACAS) (Cassar et al. 2012, Bytnerowicz et al. 2019). ARACAS, like the standard acetylene reduction assay (Hardy et al. 1968), uses the rate of acetylene ($C_2H_2$) reduction to ethylene ($C_2H_4$) as a proxy for nitrogenase activity, but provides continuous (~1 $s^{-1}$) measurements with much greater precision than measurements on a gas chromatograph (Cassar et al. 2012, Bytnerowicz et al. 2019). Immediately after extracting larvae from their food source, we placed them individually into a sealed mason jar, which we submerged in a water bath at a constant temperature of 25 °C. We connected the jar with plastic tubing to a Picarro G2106 (Santa Clara, CA) ethylene analyzer and an LI-COR LI-6262 (Lincoln, NE) $H_2O/CO_2$ analyzer. We synthesized acetylene gas in the laboratory by reacting calcium carbide ($CaC_2$) with water. We used a syringe to remove 10 ml of air from the mason jar and to inject 6 ml of acetylene gas, and allowed the air pressure to equilibrate, resulting in a total acetylene concentration of 2%, which is optimal for our ARACAS setup, and below the concentration threshold at which acetylene is explosive (Bytnerowicz et al. 2019). We ran each assay for 30–60 min, monitoring ethylene and $CO_2$ gas production continuously. In other work in our lab (Bytnerowicz et al. 2019, 2022, and ongoing work), we use this same system to study N fixation rates by bacteria in symbiosis with vascular plants. They produce ethylene when acetylene is added, as has been observed in myriad studies in the N fixation literature. The rates of ethylene production vary according to the rate of nitrogenase activity.

To test for ethylene production in the absence of acetylene, we ran control assays for *C. piceus* larvae ($N$ = 6) by placing a larva into a mason jar without any acetylene gas, and recording ethylene production continuously for 15 min. No ethylene production was detected in the absence of acetylene for all control assays. We also ran blank assays ($N$ = 8) for 1 h in which acetylene was injected into an empty mason jar, to ensure that any ethylene gas detected during the assays was being produced by the organism and not by some other means. No ethylene production was detected for all blank assays. All larvae were massed immediately after the assay had concluded, and we used whole body fresh weight (rather than dry weight) to calculate the final rate of ethylene production, as is

standard in studies of insect N fixation (e.g., Morales-Jiménez et al. 2009, Shelef et al. 2013, Ayayee et al. 2014).

Bytnerowicz et al. (2022) found that N fixation in plant-bacterial symbioses declined over the course of hours when assays were run at high temperatures. To assess for potential changes in N fixation activity over time in *C. piceus* larvae, we recorded the rate of nitrogenase activity and $CO_2$ flux over 2–4 h at constant temperatures (controlled by water bath, as above). We ran these assays with individual larvae at 20 °C ($N$ = 2), 25 °C ($N$ = 3), and 30 °C ($N$ = 2), to determine whether temperature impacted the rate at which nitrogenase activity changed over time, using different larvae for each assay.

Given that nitrogenase activity is generally considered to be saturating at an acetylene concentration of 10% and that saturating activity is the biologically relevant measure for N fixation (given the abundance of $N_2$ gas), we calculated the substrate-saturated rate of ethylene production ($V_{max}$) from measurements made at 2% acetylene [following Bytnerowicz et al. (2019)]. The substrate-saturated rate of ethylene production, which corrects for the subsaturating concentration of acetylene as well as the (small) gas leak out of the chamber, is:

$$V_{max}(t) = \left[\frac{dE(t)}{dt} + k_{eff}E(t)\right] \times \left[\frac{K_m}{A(0)\,e^{-k_{eff}t}} + 1\right], \quad (1)$$

where $V_{max}(t)$ is the saturated rate of ethylene production at time ($t$), $\frac{dE(t)}{dt}$ is the rate of ethylene change per time, $E(t)$ is the ethylene concentration at time ($t$), $k_{eff}$ is the rate at which gas leaks out of the chamber, $K_m$ is the half-saturation constant, and $A(0)$ is the concentration of ethylene at time 0. To calculate $\frac{dE(t)}{dt}$, we discarded all data recorded in the first 15 min. of each assay, to allow sufficient time for all gases in the chamber to equilibrate, and any data recorded after 30 min, to provide a consistent timeframe, and then ran a linear regression on the raw ethylene production data. $K_m$ is estimated by running multiple assays with a single individual at different acetylene concentrations. However, since preliminary data suggested that ethylene production declines rapidly following the removal of *C. piceus* from its natural habitat, it was not possible to run multiple assays using the same larva. Therefore, we used the average of 4 $K_m$ values calculated for the N-fixing symbionts of the plant species *Alnus rubra, Gliricidia sepium, Morella cerifera,* and *Robinia pseudoacacia* by Bytnerowicz et al. (2019), yielding a value of 1.975. The leak rate, $k_{eff}$, was calculated by running blank assays ($N$ = 8) for 1 h in which ethylene was injected into an empty mason jar, as in Bytnerowicz et al. (2019), yielding a mean value of 0.0032 ± 0.0054 ppb/h (uncertainty expressed as standard error).

## $^{15}N_2$ Incubations

To validate the results of ARACAS, we conducted $^{15}N_2$ incubations on *C. piceus* larvae ($N$ = 15). Directly after extracting larvae from their food source, we incubated individuals in a 60 ml plastic syringe with an atmosphere of 18.6 atom % $^{15}N_2$-labeled gas (Cambridge Isotope Laboratories, Tewksbury, MA) for 1 h at a constant temperature of 25 °C (controlled by water bath, as above). We then dried the larvae at 75 °C for a minimum of 72 h, and removed the head of each larva from the body. This was done to provide a reference point for isotopic analysis, because we assumed that the abundance of N-fixing bacteria would be minimal in the region of the head, and that no newly-fixed N would accumulate in the head tissues within the 1-h incubation period. This methodology accounts for both potential $^{15}N$-labeled ammonium and nitrate contamination in the $^{15}N_2$ gas used for the incubations (Dabundo et al. 2014), and for potential variation in natural abundance levels of $^{15}N_2$ between individual beetle larvae. We ground the head and body tissues separately with a pestle and mortar, and placed a sample of the resulting material into individual tin capsules. The samples were analyzed for $^{15}N$ content via an Isotope Ratio Mass Spectrometer at the UC Davis Stable Isotope Facility.

## Statistical Analysis

We conducted all statistical analysis using R version 3.6.2 (R Core Team 2019). We performed an analysis of variance (ANOVA) and post-hoc Tukey HSD tests at a 95% confidence level to determine whether measured rates of ethylene production in *C. piceus* larvae were significantly different from control assays, in which larvae were assayed in the absence of acetylene gas. We performed a *t*-test at a 95% confidence level to determine whether rates of N fixation measured by $^{15}N_2$ incubations were significantly different from zero.

To determine the rate ($K_{fix}$) at which N fixation in *C. piceus* declines over time, we first discarded all data recorded in the first 15 min. of each assay, to allow sufficient time for all gases in the chamber to equilibrate, and performed a moving linear regression on the raw ethylene data using a 100-point window (~1 min 40 s) at 50-point intervals (~50 s) to characterize the changing rate of nitrogenase activity over time. We converted the rates calculated from the raw ethylene data to $V_{max}$ as described above.

We then constructed a simple Bayesian model, using a modified exponential decay function, which models $V_{max}(t)$ (nmol $C_2H_4$ $g^{-1}$ $h^{-1}$) as a function of time (h). Visual analysis of preliminary results suggested an effect of temperature on the rate of decline in N fixation over time, with higher temperatures resulting in a faster decline, as observed in N fixation in plant-bacteria symbioses (Bytnerowicz et al. 2022). We, therefore, incorporated temperature and an additional parameter ($a_{fix}$) into the model, using the following equation:

$$\%fix_{ij} = 100 \times exp^{\left(-K_{fix} \times t_j \times T_i^{a_{fix}}\right)}\#, \quad (2)$$

Where $\%fix_{ij}$ is a prediction of the percentage of the initial value of $V_{max}$, which was in nmol $C_2H_4$ $g^{-1}$ $h^{-1}$, at temperature $T_i$ and time $t_j$, $K_{fix}$ is the decay constant, $t$ is the time (hr) since the assay began, $T$ is the temperature (°C) at which the assay was run, and $a_{fix}$ is a fitted parameter that allows for different rates of decline at different temperatures. The data $\%fix_{ij}$ were assumed to follow a normal distribution, with a mean given by Eq. (2) and a standard deviation $\sigma_{fix}$. The parameter $K_{fix}$ was given a vague gamma-distributed prior, and the parameters $a_{fix}$ and $\sigma_{fix}$ were given vague uniform-distributed priors.

In addition to the decline in nitrogenase activity, we also investigated the decline in respiration. To determine the rate ($K_{resp}$) at which respiration in *C. piceus* declines over time, we performed a moving linear regression on the raw $CO_2$ data using a 300-point window (~5 min) at 60-point intervals (~1 min) to characterize the changing rate of respiration over time. We then adjusted Eq. (2) by introducing an additional parameter ($b$) to account for the fact that respiration rates usually plateaued well above zero, using the following equation:

$$\%resp_{ij} = (100 - b) \times exp^{\left(-K_{resp} \times t_j \times T_i^{a_{resp}}\right)} + b\#, \quad (3)$$

Where $\%resp_{ij}$ is a prediction of the percentage of the initial rate of $CO_2$ production, which was in µmol $h^{-1}$, at temperature $T_i$ and time $t_j$, $K_{resp}$ is the decay constant, $t$ is the time (h) since the assay began, $T$ is the temperature (°C) at which the assay was run, $a_{resp}$ is a fitted parameter that allows for different rates of decline at different temperatures, and $b$ is a fitted parameter that allows respiration to plateau above zero. The data $\%resp_{ij}$ were assumed

to follow a normal distribution, with a mean given by Eq. (3) and a standard deviation $\sigma_{resp}$. The parameter $K_{resp}$ was given a vague gamma-distributed prior, and the parameters $a_{resp}$, $b$, and $\sigma_{resp}$ were given vague uniform-distributed priors.

For both models, the posterior distributions of the parameters were estimated using Markov Chain Monte Carlo (MCMC) algorithms in the "rjags" package (Plummer 2019). We ran 3 chains for 10,000 iterations each, discarding the first 1,000 iterations as burn-in, and thinning by 1 in every 10 iterations. Trace plots were inspected visually, and both Gelman and Rubin (Gelman and Rubin 1992) and Raftery and Lewis (Raftery and Lewis 1995) diagnostics were conducted, to ensure that the chains had converged.

## Results

### Nitrogenase Assays

The rate of ethylene production ($V_{max}$) for *C. piceus* larvae ranged from 8.9 to 637.1 nmol $C_2H_4$ $g^{-1}$ $h^{-1}$, with a mean rate of 258.2 ± 25.7 nmol $C_2H_4$ $g^{-1}$ $h^{-1}$ (uncertainty expressed from here onwards as standard error) (Fig. 1). These results were significantly different from zero (one-sample *t*-test: $t = 10.22$; df = 36; $P < 0.0001$), and significantly different from control data in which ethylene production was measured in the absence of acetylene (Tukey HSD: $t = 10.16$; df = 36; $P < 0.001$). No ethylene production was detected in the absence of acetylene for all control assays.

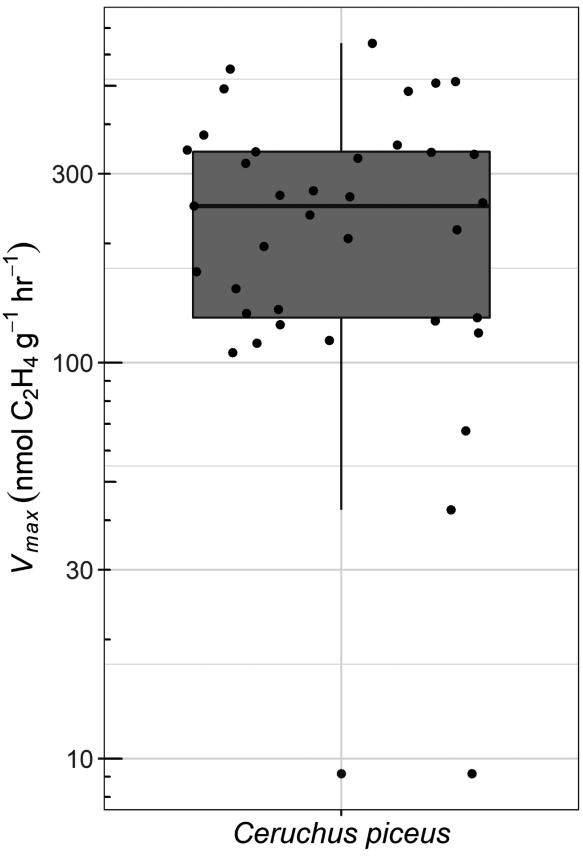


**Fig. 1.** Rates of ethylene production ($V_{max}$) in *Ceruchus piceus* larvae ($N = 37$). The boxplot displays the median and the 1st and 3rd quartiles. Whiskers indicate maximum and minimum data values within the interquartile range (IQR) multiplied by 1.5. Points are jittered horizontally for visual clarity. Note the logarithmic scale on the y axis.

The rate of ethylene production in *C. piceus* larvae declined over time after removal from their habitat and the rate of decline increased with temperature. According to our statistical model fits, at a constant temperature of 20 °C, N fixation declined by 32% per hour, whereas at 25 °C, N fixation declined by 64% per hour, and at 30 °C, N fixation declined by 89% per hour (Fig. 2, Table 1).

Respiration in *C. piceus* larvae also declined over time after removal from their habitat. According to our statistical model fits, at a constant temperature of 20 °C, respiration declined by 30% over the first hour, whereas at 25 °C, respiration declined by 56% over the first hour, and at 30 °C, respiration declined by 67% over the first hour (Fig. 3, Table 2). Unlike the decline in nitrogenase activity, respiration remained well above 0, approaching a plateau at 31% of its initial value.

### $^{15}N_2$ Incubations

The N content of larval samples was calculated for the larval body (minus the head capsule, which provided a reference point for isotopic analysis), and ranged from 4.53 to 8.16% (100*g N $g^{-1}$ dry mass), with a mean value of 5.99 ± 0.32%. The isotopic labeling elevated larval body tissue to 5.05 ± 1.80 ‰ $\delta^{15}N$, compared to –1.07 ± 0.27 ‰ $\delta^{15}N$ for larval head tissue (Fig. 4). According to the isotopic labeling method, the rate of N fixation in *C. piceus* larvae ranged from –0.16 to 21.51 µg N g dry larval body tissue$^{-1}$ $h^{-1}$, with a mean rate of 6.6 ± 1.8 µg N g dry larval body tissue$^{-1}$ $h^{-1}$, which was significantly greater than zero (one-sample t-test: $t = 3.78$; df = 14; $P = 0.001$). Although it is not possible for a negative rate of N fixation to occur, it is possible to obtain negative estimates from isotopic analysis due to natural variation in the isotopic makeup of samples. In the case of 1 of our samples, for which a rate of –0.16 µg N g dry larval body tissue$^{-1}$ $h^{-1}$ was calculated, it is likely that this larva was fixed at a zero or near-zero rate.

## Discussion

In this study, we documented, for the first time, that N fixation occurs within the larvae of *Ceruchus piceus*. Additionally, during the course of our work we found that N fixation declines rapidly after *C. piceus* is removed from its natural habitat. In the following sections, we explore how these results compare to other findings and what they mean for the role of N fixation in beetle nutrition. We also discuss potential implications for both past and future studies on N fixation in insects.

### Rates and Variation of N Fixation Within *C. piceus* Larvae

The rates of ethylene production that we measured in *C. piceus* larvae (mean of 258.2 nmol $C_2H_4$ $g^{-1}$ $h^{-1}$) were considerably higher than most rates which have been reported for other beetle species in the existing literature. For example, Ayayee et al. (2014) reported ethylene production rates of 0.58 ± 0.23 nmol $C_2H_4$ $g^{-1}$ $h^{-1}$ in larvae of the cerambycid beetle *Anoplophora glabripennis*, Kuranouchi et al. (2006) reported ethylene production rates of 1.25 ± 0.37 nmol $C_2H_4$ $g^{-1}$ $h^{-1}$ in larvae of the stag beetle *Dorcus rectus*, and Shelef et al. (2013) reported ethylene production rates of 3.135 ± 0.7 nmol $C_2H_4$ $g^{-1}$ $h^{-1}$ in larvae of the weevil *Conorbynchus pistor*. A couple of studies report rates much higher than our results, although unit conversion does introduce sources of error. For example, Behar et al. (2005) reported rates of 18.0 ± 1.5 nmol $C_2H_4$ insect$^{-1}$ $h^{-1}$ in the medfly *Ceratatis capitata*, which equates to 3,314.92 nmol $C_2H_4$ $g^{-1}$ $h^{-1}$ if the average mass of *C. capitata* is assumed to be

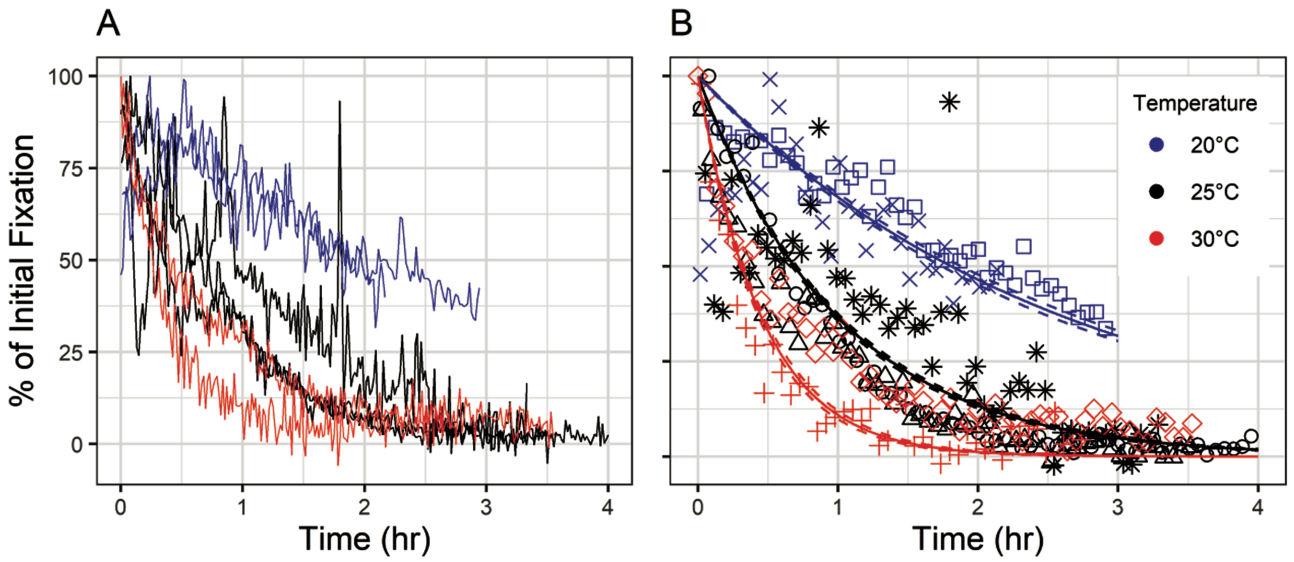


**Fig. 2.** A) Decline in N fixation in *C. piceus* larvae after removal from their habitat. The vertical axis shows the percentage of initial N fixation rather than total N fixation to facilitate the comparison across individuals with different initial rates. Data are shown for 3 separate temperatures: 20 °C (blue), 25 °C (black), and 30 °C (red). Each line represents 1 individual larva. B) Statistical fits to the data in (A). Both mean values (solid curves) and 95% confidence intervals (dashed curves, representing SE) are shown, overlaying a 25% subset of the model data. Different shapes represent individual larvae.

**Table 1.** Estimates for the mean, standard deviation, and 95% confidence interval for the $K_{fix}$, $a_{fix}$, and $\sigma_{fix}$ parameters for the model of the decline in N fixation in *C. piceus* larvae over time

| Parameter | Mean | SD | 2.5% | 97.5% |
|---|---|---|---|---|
| $K_{fix}$ | 0.0189 | 0.00142 | 0.0162 | 0.0189 |
| $a_{fix}$ | 4.34 | 0.0856 | 4.18 | 4.34 |
| $\sigma_{fix}$ | 10.5 | 0.197 | 10.1 | 10.5 |

5.43 mg (Valtierra-de-Luis et al. 2019). Similarly, Morales-Jiménez et al. (2009) reported rates of 15.96 ± 2.22 nmol $C_2H_4$ insect$^{-1}$ h$^{-1}$ in the red turpentine beetle *Dendroctonus valens*, which equates to 3,627.27 nmol $C_2H_4$ g$^{-1}$ h$^{-1}$ if the average mass of *D. valens* is assumed to be 4.4 mg (Liu et al. 2011) [see Bar-Shmuel et al. (2020) for an up-to-date, comprehensive review of insect N fixation studies]. It is possible that the unusually high rates of N fixation reported in some studies are due, at least in part, to methodological error. Behar et al. (2005) report that rates of ethylene production detected in *C. capitata* were significantly different from control assays when acetylene was injected into an empty jar, however, they still detected 6.0 ± 2.9 nmol $C_2H_4$ insect$^{-1}$ h$^{-1}$ (over 1,000 nmol $C_2H_4$ g$^{-1}$ h$^{-1}$ if the average mass of *C. capitata* is 5.43 mg) in control assays, which appears to be very high. Reporting rates per individual insect, rather than per gram, may also introduce error as it fails to control for differently-sized larvae.

Rates of ethylene production in *C. piceus* varied considerably, from 8.9 to 637.1 nmol $C_2H_4$ g$^{-1}$ h$^{-1}$. Although our sample size was modest (37 larvae), it is substantially higher than most previous studies on N fixation in insects. For example, Ayayee et al. (2014) assayed 6 individuals of *Anoplophora glabripennis*, Kuranouchi et al. (2006) assayed 8 individuals of *Dorcus rectus*, and Behar et al. (2005) conducted 3 replicate assays with multiple (12–17) individuals of the fruit fly *Ceratitis capitata* per assay. Incubating multiple insects per assay would also underestimate individual variation. Rates of N fixation in insects may vary to a much greater extent than we currently assume, and it is evident that future studies should involve large sample sizes to ensure that the results accurately represent variation within species.

There are a variety of plausible drivers of the variation we observed in N fixation rates. The structure and bacterial community of an insect's gut can alter dramatically as it matures from larva to adult (Nardi et al. 2006), and some studies have found that adult insects fix N at lower rates than larvae (Peklo and Satava 1949, Morales-Jiménez et al. 2009, Shelef et al. 2013), potentially due to the fact that adults have lower dietary N requirements. Benemann (1973) also found that worker termites produced ethylene at much higher rates than reproductive termites or soldiers. This suggests that N fixation may be impacted by an individual's instar, age, weight, or caste. It has also been theorized that N fixation may arise more frequently in social insects due to horizontal transfer of endosymbionts leading to more stable bacterial communities (Kneip et al. 2007). Some studies have demonstrated that dietary N availability impacts rates of N fixation, with higher dietary N availability being correlated with decreased rates of N fixation (Breznak et al. 1973, Meuti et al. 2011), just as some plant species are known to regulate N fixation in response to soil N availability (Menge et al. 2022). N fixation is an enzymatic process which is affected by temperature in plants (Bytnerowicz et al. 2022), and it is likely that variation in the temperature, moisture, or other biophysical conditions of the environment would affect N fixation in insects. A diversity of potential N-fixing bacterial species has been identified within the guts of insects (Behar et al. 2005, Morales-Jiménez et al. 2012, Mohammed et al. 2018) and it is not yet known how different bacterial species or community compositions might affect rates of N fixation. Further research is required to determine the extent to which each of these factors may contribute to individual variation.

## Implications of the Decline in N Fixation When Removed From Their Natural Habitat

When we removed *C. piceus* larvae from the decaying logs which comprise their natural habitat and placed them into an artificial laboratory environment, we found that measured rates of ethylene

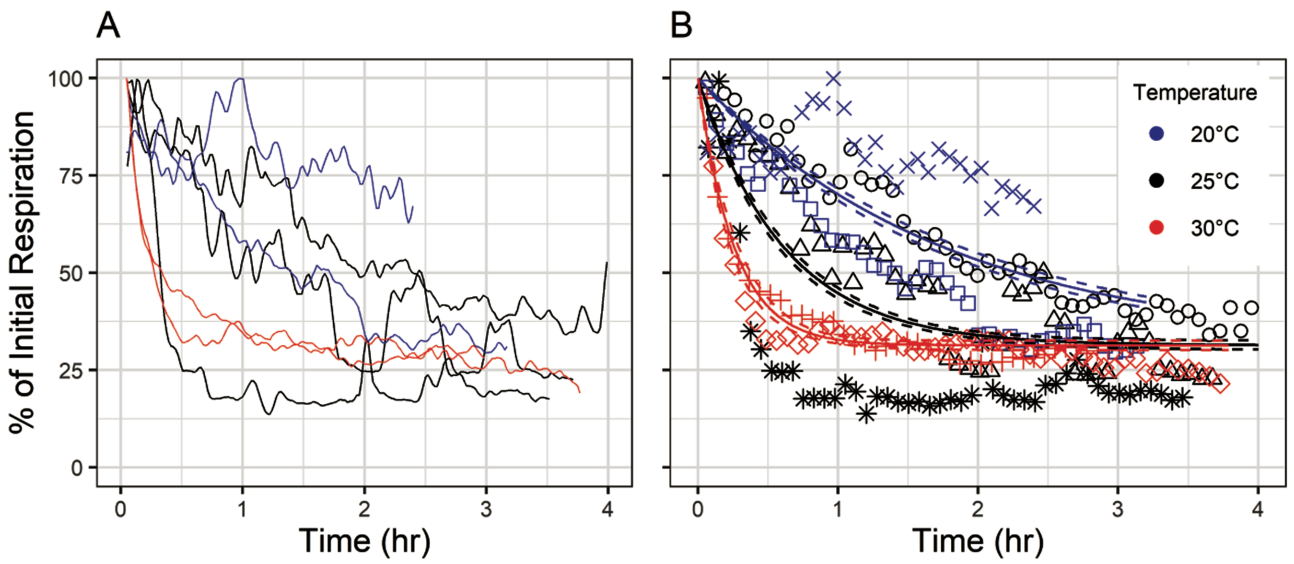


**Fig. 3.** A) Decline in respiration in *C. piceus* larvae after removal from their food source. The vertical axis shows the percentage of initial respiration rather than total respiration to facilitate the comparison across individuals with different initial rates. Data are shown for 3 separate temperatures: 20 °C (blue), 25 °C (black), and 30 °C (red). Each line represents 1 individual larva. B) Statistical fits to the data in (A). Both mean values (solid curves) and 95% confidence intervals (dashed curves, representing SE) are shown, overlaying a 25% subset of the model data. Different shapes represent individual larvae.

**Table 2.** Estimates for the mean, standard deviation, and 95% confidence interval for the $K_{resp}$, $a_{resp}$, $b$, and $\sigma_{resp}$ parameters for the model of the decline in respiration in *C. piceus* larvae over time

| Parameter | Mean | SD | 2.5% | 97.5% |
|---|---|---|---|---|
| $K_{resp}$ | 0.0218 | 0.00324 | 0.016 | 0.0286 |
| *aresp* | 4.73 | 0.182 | 4.38 | 5.09 |
| *b* | 31.3 | 0.612 | 30.1 | 32.5 |
| $\sigma_{resp}$ | 13.9 | 0.278 | 13.4 | 14.5 |

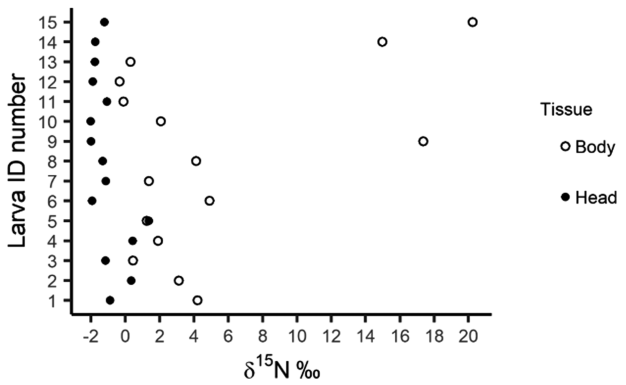


**Fig. 4.** Isotopic signal of nitrogen in head (closed circles) and body (open circles) tissue of *Ceruchus piceus* larvae ($N$ =15). As is standard, we express the isotopic content as $\delta^{15}$N per mil relative to air (see Methods). Each row is a different individual.

production declined over time. This decline appeared to be rapid: at 25 °C, N fixation dropped by 50% in 41 min. Temperature also impacted the rate of decline, with higher temperatures causing a faster rate of decline: at 30 °C, N fixation dropped by 50% in 18 min. Although our sample size was small (7 larvae spread across 3 different temperature treatments), our data show a clear and consistent trend. The precise causes of the decline through time and the faster decline at higher temperatures are unclear, though it is notable that respiration also declined over time: our model demonstrates that respiration can drop by 50% in 47 min at 25 °C. Although these 2 models do not provide a perfect comparison, since ethylene production represents bacterial activity whereas $CO_2$ production represents combined larval and bacterial activity, they do support the idea that a decline in N fixation is related to a decline in metabolic function.

A decline in metabolic function could occur as a result of potential stress factors. Invertebrates show similar stress responses to vertebrates – the fight or flight response causes resources to be reallocated away from non-essential functions (Adamo 2012). In this particular study, *C. piceus* larvae were inadvertently subjected to numerous environmental stressors during the assay process, such as changes in temperature, light, different moisture conditions, and potential mechanical stress from being handled. They also experienced food deprivation, as a direct result of being removed from the decaying logs on which they were feeding. It is unlikely that the larvae would have completely emptied their digestive systems during the 2–4 h period in which the assays were conducted (Songvorawit et al. 2022), however, gut conditions would have likely been altered in sub-optimal ways. Without further experimentation, it is very difficult to disentangle the specific effects of each of these factors, or to determine whether they directly impacted the N-fixing bacteria by changing conditions in the larval hindgut, or indirectly by inducing a stress response in the larva. More research is needed to understand how laboratory conditions affect N fixation in insects and how methods can be adapted to minimize this impact.

The decline in N fixation after removal from their habitat has considerable implications for studies of N fixation in insects. As early as 1980, Prestwich et al. (1980) found that if termites from the genera *Nasutitermes* and *Rhynchotermes* were removed from their colonies and held under laboratory conditions for 2 days, measured N fixation dropped to a very small fraction of its original value. The authors concluded that their work highlighted 'the importance of conducting future $N_2$ fixation studies under field conditions', and Prestwich and Bentley (1981) went on to develop a method for measuring N fixation rates in entire colonies of *Nasutitermes*

*corniger* in the field. Interestingly, termites that were held in the lab overnight were housed inside whole, intact nests that had been collected from the field, stored inside a plastic bag (Prestwich et al. 1980), which suggests that food deprivation was not necessarily the cause of the observed decline in N fixation. Curtis and Waller (1995) also found that N fixation in termites of the family *Rhinotermitidae* was negatively affected by laboratory conditions, even though the termites were housed with their original food source. Although the termites could regain or even exceed their original rate of N fixation, this could take 2–4 mo, and depending on initial environmental conditions.

Despite this decades-old knowledge, N fixation in insects is still primarily measured under laboratory conditions – specimens are usually assayed inside glass vials with no natural substrate. Measurements are frequently taken long after the insects have been removed from their natural habitat, and repeated measurements over time are not common. For example, Citernesi et al. (1977) assayed a scarabaeid beetle of the genus *Cetonia* for 24 h, Behar et al. (2005) assayed individuals of *Ceratitis capitata* at 30 °C for 5 h, and Ayayee et al. (2014) assayed individuals of *Anoplophora glabripennis* at 28 °C for 24 h. However, Kuranouchi et al. (2006) took repeated measurements over a 4 h period when assaying individuals of *Dorcus rectus*, and found consistent rates of ethylene production throughout the 4 h period, suggesting that the decline in N fixation over time we observed may not be universal across insect species and/or measurement conditions. Nevertheless, it is quite possible that the rates of ethylene production reported in many studies are much lower than the true value, and may explain why the results from our study were higher than many previously recorded results.

It is worth noting that this rapid decline in N fixation over time likely impacts our own recorded rates of ethylene production for *C. piceus*. We conducted $V_{max}$ calculations on data recorded between 15 and 30 min. from the start of each assay, and our model suggests that N fixation could have already declined by 22–40% during this timeframe at 25 °C. It is therefore highly possible that the true rate of N fixation in *C. piceus* larvae could be even higher than the rates we have reported in this study.

### Implications for Nutritional Ecology and Ecosystem-Level N Cycling

Our results are potentially of significance to insect nutritional ecology. Xylophagous insects face a significant stoichiometric imbalance between their diet and their body tissues (Filipiak and Weiner 2014). Although some studies have demonstrated that xylophagous insects can overcome dietary N deficiency through associations with specialized fungi (Ayres et al. 2000) or gut microbes that can synthesize essential amino acids (Ayayee et al. 2016), numerous studies have speculated that N fixation plays a key role in insect nutritional ecology (Behar et al. 2005, Douglas 2009, Morales-Jiménez et al. 2012). Few studies have directly measured the assimilation of fixed N into host tissues (Fujita and Abe 2006, Meuti et al. 2011), therefore it is difficult to say with certainty that associating with N-fixing bacteria directly benefits the host. (Douglas (2009) even suggests that the resulting ammonium could be potentially toxic to the host.) If future studies are able to clearly demonstrate that an association with N-fixing bacteria produces an increase in insect fitness, our results suggest that N fixation could be a much more significant source of dietary N to xylophagous insects than previously thought.

Our results also have the potential to alter the current thinking regarding how insect-mediated N fixation operates at an ecosystem scale. Previous attempts have been made to estimate the contribution of insect N fixation to the ecosystem, but efforts have primarily focused on termites in tropical regions. For example, Yamada et al. (2006) estimated that termites in tropical forests in Thailand may contribute approximately 0.21–0.28 kg N $ha^{-1}$ $y^{-1}$, accounting for 7–22% of biological N fixation. Studies that focus on N fixation in temperate forests, and particularly on beetle species, have generally concluded that insect N fixation is likely to be insignificant at the ecosystem level (Nardi et al. 2002, Tanahashi et al. 2018). If, as our results suggest, previous studies have been systematically and severely under-reporting rates of N fixation in insects, then insect N fixation might be more important at the ecosystem scale than previously thought. Even if insect N fixation does not comprise a large proportion of total biological N fixation, it could still have a significant impact on various ecosystem processes by providing localized N inputs. Additionally, N fixation may allow insects to utilize food sources that would otherwise be unavailable to them, further impacting ecosystem processes. Evidently, much research is still needed to understand how insect N fixation may impact decomposition, nutrient cycling, pest outbreaks, and community composition, and to determine whether insect N fixation may provide a partial explanation for the discrepancies seen in N budgets of forested ecosystems (Johnson and Turner 2014).

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Isobel Mifsud (Conceptualization-Equal, Formal analysis-Lead, Funding acquisition-Lead, Investigation-Lead, Methodology-Equal, Project administration-Lead, Resources-Equal, Visualization-Lead, Writing – original draft-Lead, Writing – review & editing-Equal), Palani R. Akana (Formal analysis-Supporting, Investigation-Supporting, Methodology-Supporting, Resources-Supporting, Writing – review & editing-Equal), Thomas Bytnerowicz (Formal analysis-Supporting, Methodology-Supporting, Resources-Supporting, Software-Lead, Writing – review & editing-Equal), Steve Davis (Conceptualization-Equal, Methodology-Equal, Writing – review & editing-Equal), Duncan Menge (Conceptualization-Equal, Formal analysis-Supporting, Methodology-Equal, Resources-Equal, Writing – review & editing-Equal)

## Data Availability

Available on request.

## Supplementary Material

Supplementary material is available at *Environmental Entomology* online.

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