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*CORRESPONDENCE
Oliver M. Beckers
✉ obeckers@murraystate.edu

Phenotypic plasticity of male calls in two populations of the katydid *Neoconocephalus triops* (Insecta: Tettigoniidae)

Oliver M. Beckers[1]*, Teiya Kijimoto[2] and Johannes Schul[3]

[1]Department of Biological Sciences, Murray State University, Murray, KY, United States, [2]Division of Plant and Soil Sciences, West Virginia University, Morgantown, WV, United States, [3]Division of Biological Sciences, University of Missouri, Columbia, MO, United States

The ability to respond to environmental changes plays a crucial role for coping with environmental stressors related to climate change. Substantial changes in environmental conditions can overcome developmental homeostasis, exposing cryptic genetic variation. The katydid *Neoconocephalus triops* is a tropical species that extended its range to the more seasonal environment of North America where it has two reproductive generations per year. The harsher winter conditions required adults to diapause which resulted in substantially different mating calls of the diapausing winter animals compared to the non-overwintering summer animals in northern Florida. The summer call corresponds to that of tropical populations, whereas the winter call represents the alternative call phenotype. We quantified call plasticity in a tropical (Puerto Rico) and a temperate population of *N. triops* (Florida) that differ in experiencing winter conditions in their geographic regions. We hypothesized that the plastic call traits, i.e., double-pulse rate and call structure, are regulated independently. Further, we hypothesized that phenotypic plasticity of double-pulse rate results in quantitative changes, whereas that of call structure in qualitative changes. We varied the photoperiod and duration of diapause during male juvenile and adult development during rearing and analyzed the double-pulse rate and call structure of the animals. Double-pulse rate changed in a quantitative fashion in both populations and significant changes appeared at different developmental points, i.e., the double-pulse rate slowed down during juvenile development in Florida, whereas during adult diapause in Puerto Rico. In the Florida population, both the number of males producing and the proportion of total call time covered by the alternative call structure (= continuous calls) increased with duration spent in diapause. In the Puerto Rico population, expression of the alternative call structure was extremely rare. Our results suggest that the expression of both pulse rate and call structure was quantitative and not categorical. Our systematic variation of environmental variables demonstrated a wide range of phenotypic variation that can be induced during development. Our study highlights the evolutionary potential of hidden genetic variation and phenotypic plasticity when confronted with rapidly changing environments and their potential role in providing variation necessary for communication systems to evolve.

KEYWORDS

homeostasis, signal evolution, communication, diapause, development

Introduction

In the foreseeable future, climate change will expose many organisms to different or more extreme environmental conditions than they have experienced during their recent evolution. Their evolutionary trajectory will depend on their capacity to respond to these changes (Seebacher et al., 2015; McInerny and Etienne et al., 2012) in terms of persistence in the geographic region or colonization of new regions (Agrawal, 2001). Understanding evolutionary responses to the rapidly changing environment will be a major challenge for evolutionary and environmental biology in the coming decades (sensu Bozinovic and Pörtner, 2015).

Most organisms have been selected to maintain their phenotype over the range of environmental conditions that they commonly experience (e.g., physiological and developmental homeostasis; review in Debat and David, 2001). Compensatory mechanisms may accommodate less optimal genetic alternatives under common conditions and thus preserve cryptic genetic variation (West-Eberhard, 2005). However, when organisms experience environmental conditions that exceed the capacity of homeostasis, cryptic genetic variability may be revealed as alternative phenotypic variants (= phenotypic plasticity; West-Eberhardt, 2003; Paaby and Rockman, 2014). If the exposed alternative phenotypes are adaptive, such phenotypic plasticity may lead to rapid evolutionary change and in extreme cases the selected trait may even become dominant in a population within a single generation (Campbell-Staton et al., 2017).

Here, we characterize call plasticity of the reproductive communication system in the tropical katydid *Neoconocephalus triops* (Orthoptera, Tettigoniidae) that extended its range into the novel environment of temperate North America (Greenfield, 1990). This katydid occurs through most of the American tropics and Caribbean, extending into temperate regions as far North as Ohio and Missouri (Whitesell, 1974; Walker and Greenfield, 1983; Greenfield, 1990). Its life history is typical for tropical katydids with direct egg development (i.e., without egg diapause) and multiple generations per year. In Puerto Rico, *N. triops* is multivoltine and males call all year long (Greenfield, 1990). In temperate regions, *N. triops* is bivoltine with one generation reproducing in the fall and their offspring overwintering as adults and reproducing in the following spring (Whitesell, 1974). Each generation of both tropical and temperate *N. triops* populations are largely the offspring of the immediately preceding generation, i.e., the two generations do not form separate gene pools (Whitesell, 1974).

Calls of tropical males consist of rapidly repeated pulse pairs or double-pulses (~110-120 double-pulses/s at 25°C; Beckers and Schul, 2010) that are grouped into rhythmically repeated chirps of about 1s duration (see Methods for detailed description). The double-pulse rate is the most important temporal call feature used in species recognition in *N. triops* (Beckers and Schul, 2008) and some tropical populations of *N. triops* are substantially more attracted to chirped than continuous calls (Beckers, 2008). In temperate regions, calls from the non-overwintering generation (fall) are comparable to those of tropical populations, i.e., the calls have a similar double-pulse rate and are structured in chirps (Whitesell and Walker, 1978; Beckers and Schul, 2008). Calls of

overwintering generations, however, differ substantially from those of the non-overwintering generation: The double-pulse rate is about 20% slower at the same ambient temperature and the calls are typically categorized as continuous (Whitesell and Walker, 1978; Beckers and Schul, 2008). This 'alternative call' differs so strikingly from the 'standard call' of tropical and non-overwintering males that the overwintering generation was initially described as a different species (i.e., *N. fuscostriatus*; Whitesell, 1969; Walker and Greenfield, 1983). The differences between standard and alternative calls in chirp structure and pulse rate each are equivalent to species defining differences among other *Neoconocephalus* species (Schul et al., 2013).

The short photoperiod experienced by overwintering adults is a critical factor for the induction of the alternative phenotype in the temperate population (Whitesell and Walker, 1978; Beckers and Schul, 2008). When reared under the conditions that reliably result in the alternative phenotype in temperate *N. triops*, even tropical (Puerto Rico, Costa Rica) *N. triops* males expressed similarly reduced pulse rates (Beckers and Schul, 2010). However, neither the environmental conditions controlling call plasticity, nor the possible phenotypic variability have been systematically quantified.

The environmental conditions that *N. triops* experienced during its range expansion likely selected for traits supporting overwintering (e.g., frost tolerance, adult diapausing stages) while maintaining synchrony between male and female reproductive periods. We previously concluded that these traits necessary for survival likely had pleiotropic effects that resulted in the expression of the alternative call type as a by-product rather than an adaptation (Beckers and Schul, 2010). As a result, female preferences likely evolved to accommodate this male call plasticity for communication to remain intact in both generations in the temperate environment (Beckers and Schul, 2010).

In this study, we quantified phenotypic plasticity in double-pulse rate and call structure in two populations of *N. triops*. One population expresses call phenotypic plasticity in the field (Florida; Whitesell and Walker, 1978), and the other one does not (Puerto Rico; Walker and Greenfield, 1983; Greenfield, 1990). We chose these two populations because they represent the transition from continuously breeding, multivoltine *N. triops* tropical populations that do no express call plasticity (Puerto Rico) to bivoltine populations expressing call plasticity in the field (Florida) as the result of newly experienced winter conditions in temperate regions. In our experiments, we systematically varied the rearing conditions during development (i.e., time spent in diapause conditions) and measured their effects on the two call traits in the two populations. Preliminary observations (OMB unpublished data) suggest that some *N. triops* produce chirped calls with slow pulse-rates after being placed in diapause. Therefore, we hypothesized that phenotypic plasticity of double-pulse rate and chirp structure are two distinct traits, responding independently to changes in photoperiod. Calls of *Neoconcephlaus* species (SINA, 2023), and the majority of other Orthopterans (e.g., Gerhardt and Huber, 2002), are either structured in chirps or are continuous, indicating that call structure is a qualitative trait. In contrast, pulse rate varies quantitatively among acoustic insects and displays continuous variation in context of temperature changes (e.g., Gerhardt and Huber, 2002; *N. triops*: Whitesell and Walker, 1978).

We hypothesized that plasticity of call structure in *N. triops* is likely a qualitative trait, with two distinct trait versions (chirped vs continuous call) while plasticity of pulse rate is likely a quantitative trait displaying continuous variation.

Our study contributes to a better understanding of potential origins and evolutionary responses to rapid environmental change that can contribute to phenotypic variation necessary for communication signals to evolve. The reported results will serve as the basis for future studies on the neural and genetic mechanisms underlying this plasticity and call diversification.

Materials and methods

General experimental approach

Short photoperiods related to winter conditions induce differences in the mating calls of *N. triops* (Whitesell and Walker, 1978; Beckers and Schul, 2008; Beckers and Schul, 2010). We reared juveniles from Florida and Puerto Rico under winter conditions to adulthood and then transferred them for different periods of time past adult molt (= treatment groups) in diapause for up to 140 days, which corresponds approximately to the diapausing duration in northern Florida (Beckers and Schul, 2008). We recorded and compared pulse rate and call structure across diapause treatments for each population (for details, see below). In addition, we reared and recorded the calls of a short-day control group (without diapause) and a control group reared in long-day to adulthood. We also recorded Wildtype animals from both populations for reference.

Animals

Florida population

We collected about 50-80 non-diapausing males and females each during the summers of 2020 and 2021 around Gainesville, Florida, USA. We collected eggs from these animals to raise their offspring under experimental conditions (i.e., different durations spent in diapause and control conditions) in the laboratory. All experiments with the Florida population were conducted at Murray State University, Murray, Kentucky, USA.

Puerto Rico population

We collected about 50-80 non-diapausing tropical males and females each in the winter of 2016/2017 in North-Eastern Puerto Rico. These animals were used to establish a permanent culture at the University of Missouri (Columbia, Missouri, USA). All experiments with the Puerto Rico population were conducted at the University of Missouri, Columbia, Missouri, USA.

Animal care and breeding conditions

We placed 30-160 adult animals (collected in the field or lab generations derived from culture) in cages for mating and oviposition in potted grass. These cages were in climate-controlled chambers or incubators at a light/dark cycle of 15/9h with day/night temperatures of 26/22°C, respectively. Relative humidity was kept between 40 and 60%. These conditions roughly correspond to summer conditions in northern Florida and Puerto Rico and have been used in previous studies (Beckers and Schul, 2008; Beckers and Schul, 2010).

We replaced the egg-laying grass every two weeks throughout the lifespan of the females. We removed the eggs from the grass and placed them in petri dishes (90 x 15 mm, Fisher-Scientific) lined with filter paper (85 mm, Grade 1, Whatman). We kept the filter paper moist with a saturated Methylparaben (NF grade, Amresco) solution to allow for egg development and prevent fungal growth in the petri dishes (Beckers and Schul, 2008). We placed the dishes inside a closed food storage box (Tupperware) at the same conditions as the breeding animals (see above) and checked daily for the emergence of juveniles.

Hatchlings were transferred to custom-built screen cages (45 x 10 x 25cm, length x width x height, 50-100 juveniles/cage) and kept on wheat seedlings, and water gel (Tasty Worm Nutrition) inside incubators. After 8-10 weeks under short-day conditions (see below), when insects reached 4-5th instars, we separated males and females and moved them to cages at a lower density (about 15-25 per cage). We removed adult males every day (Florida) or every 3 days (Puerto Rico) from the cages and transferred them into diapause conditions (see below). All treatment and Short-day control males experienced as juveniles cycles of 11/13h light/dark at temperatures of 23/17°C, respectively. Previous work (Beckers and Schul, 2008; Beckers and Schul, 2010) and preliminary experiments demonstrated that these short-day conditions paired with diapause conditions result in the expression of the winter or alternative call phenotype. Short-day controls were reared under short-day conditions (i.e., 11/13h light/dark at 23/17°C, respectively) and Long-day controls under long-day conditions (15/9h light/dark at 26/22°C, respectively) from first instars to adulthood. Short-day controls were transferred to long-day conditions and Long-day controls were kept in long-day conditions for males to start calling. The humidity across all rearing conditions ranged between 40 and 60%.

Diapause treatments

After short-day rearing to adulthood, we transferred animals to diapause conditions. The duration males spent in diapause differed among treatment groups and ranged between 0 to 140 days. The specific treatment groups were: 0, 28, 56, 84, 112, and 140 days in diapause. Preliminary experiments showed that Florida and Puerto Rico animals differed in their adult development. When Puerto Rico animals were kept in short-day conditions, they started to call after 10-14 days, i.e., they had completely matured and were ready to reproduce. Florida animals, however, never started calling under these conditions, indicating that their adult development was arrested, as expected when in reproductive diapause. To account for these differences, we transferred adult Puerto Rico animals after 2-4 days in short-day conditions into diapause conditions in which they did not call, while Florida adult animals stayed for 14 days in

short-day conditions before being transferred to diapause conditions following a previously established rearing protocol (Beckers and Schul, 2008). Diapause conditions had cycles of 9.5/14.5h light/dark at 17/12°C, respectively.

After staying in diapause for a given treatment duration, we transferred the males to long-day conditions (light/dark cycle of 15/9h at 26/22°C, respectively) to induce final maturation and calling. While Florida males typically started calling after 3-4 weeks, Puerto Rico males often called within the first few days after being transferred to these long-day conditions.

We conducted diapause experiments with the Puerto Rico population in 2019-20 with approximately the 5th-8th generation in culture. Experiments with the Florida population took place between 2020-22 and used primarily F_1 animals (i.e., offspring from wild collected insects) and a small number of F_2 and F_3 insects.

Call recordings

Males were placed individually in small cylindrical mesh enclosures (10 cm diameter, 15 cm high) housed inside of boxes lined with acoustic foam (HFW-2 or HFW-4, Hush Foam, Silent source). Microphones were placed inside these boxes within 10 cm of the male. The boxes were placed inside temperature-controlled semi anechoic chambers. Echoes were sufficiently suppressed to not influence the measurements of pulse and chirp pattern. The individual boxes strongly reduced the crosstalk between individual male's recordings. We cannot rule out that males heard each other during recordings. Even though *N. triops* males do synchronize the beginning of their calling, i.e., they stimulate each other to start calling at the minute-scale, they do not synchronize the timing of their chirps (seconds-scale) or double-pulses (milliseconds-scale; OMB and JS personal observation). Thus, potential acoustic interaction among males would not influence the measurements in this study.

We recorded Florida males with electret tie-clip microphone (ATR3350; Audio-Technica) and digitized the signals through an 8-channel interface (Tascam US-1800) and the software Cubase 5.0SL (Steinberg) at 48kHz sampling rate and 16-bit resolution. We recorded Puerto Rico males using USB voice recorders (VR1.0; DB9PRO) at 48kHz sampling rate and 16-bit resolution. These voice recorders use 1/8" electret microphones with high sensitivity between 1-20 kHz. Recordings were inspected and trimmed for analysis using either Audacity for MacOS (Version 3.21; Audacity team) or WavePad (Audio Editor V. 16.65, NCH Software) software. We recorded all males at 25 ± 1°C ambient temperature.

Call analysis

The basic call of *N. triops* is shown in Figure 1. *Neoconocephalus triops* males produce calls with a double-pulse (DP) pattern with each DP consisting of two sets of alternating opening and closing movements of the wings (Walker, 1975), with only the closing movements producing sound pulses. The two loud pulses have different periods (i.e., duration from the beginning of the first pulse to the beginning of the second pulse), resulting in paired sound pulses (Figure 1A). These DP are grouped into rhythmically repeated chirps of roughly 1s duration that are separated by 50-100ms of silence (Figure 1B).

Double-pulse rate

We measured the time from the beginning of each DP to the beginning of the next (= DP-period, Figure 1A) of 1-3s of calling of each male, which corresponded to about 100-300 DP-periods. For the temporal measurements, we extracted the envelope from the signals while reducing the sampling rate to 12 kHz (Florida) or 8 kHz (Puerto Rico), equivalent to a temporal resolution of 0.083ms or 0.125ms, respectively. We used the custom software Song_X developed by JS to measure the DP-periods. For each animal, we calculated the average DP-period. The pulse pattern of insects or
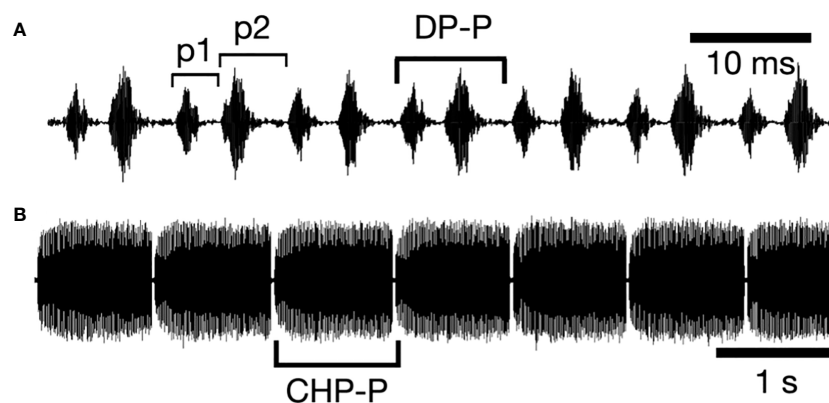

FIGURE 1
Schematic of *N. triops* male calls at the millisecond **(A)** and second **(B)** scale. **(A)** High amplitude sound pulses are produced during the closing movements of the forewings. Alternating pulse periods (p1, p2) result in a double-(DP) pulse rhythm. We measured the double-pulse period (DP-P), which is the duration between the beginning of the first pulse of a DP and the beginning of the first pulse of the next DP and converted them to DP rates (1/DP-P). **(B)** Double-pulses are grouped into chirps of about 1s duration, repeated after silent intervals of about 50ms duration. We measured chirp periods (CHP-P) from the beginning of a chirp to the beginning of the following chirp.

frog calls are typically described as pulse rates rather than pulse periods (Gerhardt and Huber, 2002). We therefore converted the DP-period of each individual to the DP-rate (= 1/DP-period) and present the data here as DP-rate (= DP/s; Figure 2).

Chirp analysis

To measure chirp periods (i.e., the duration between the beginning of a chirp and the beginning of the subsequent chirp, Figure 1B), we selected 6-8 minutes of calling for each animal. These sections typically contained multiple calling bouts of 0.5-5 minutes duration. For temporal measurements, we extracted the envelope with a sample rate of 1 kHz for analysis and used the custom software Song_X to measure the chirp periods.

At the beginning of calling bouts, a few of the chirps often had low amplitude that made it difficult to reliably measure their periods. Such chirps early in the bout were excluded from analysis. The last one or two chirps were substantially shorter than those of the rest of the bout and were excluded from the analysis as well. A calling bout ended if the silent interval between two chirps was longer than 5s. We did not include the silent periods between calling bouts in the analysis. Uninterrupted calling for longer than 5s (or 'unusually long chirps') are categorized throughout the manuscript as 'calling pieces' to distinguish this type of calling from rhythmically interrupted chirps and calling bouts (see Supplementary Materials). The distribution of chirp durations in the Puerto Rico population is tightly centered around 800-1000ms with a sharp drop off to longer chirps. Less than 20 chirps among the more than 10,000 measured chirps of this population were longer than 2.0s, only one was longer than 5.0s. The distribution of chirp periods in the Florida population was broader extending to 3s. Chirp durations beyond three seconds occurred at a low frequency; about 150 chirps (out of more than 10,000) had durations between 5 and >500s. Few chirps with durations between 3 and 8s occurred. We tried other cut-offs between 3 and 8s, which did not change the outcomes or conclusions of this analysis.

The loss of the chirp structure resulting in continuous calls (alternative structure) in 'winter males' (Whitesell and Walker, 1978; Beckers and Schul, 2008) suggests deterioration of the neural chirp pattern generator. To detect effects of this deterioration on the chirp pattern (aside from the complete loss), we used the Coefficient of Variation (or 'C.V.' = standard deviation/ mean) as a measure for the quality of the chirp pattern, i.e., low C.V. values indicate consistent chirp periods and thus a high quality of the pattern, whereas high C.V. values indicate more variability and thus a lower pattern quality. We expected that adult diapause results in reduced pattern quality (i.e., increased within-male variation and C.V.). We calculated the average chirp period and the C.V. for each individual's call recording.

We did not collect chirp data from wild katydids from Puerto Rico in 2017 but used recordings collected previously from the same population (Beckers and Schul, 2010). We used these recordings for the comparison of DP-rate with the lab-reared animals. We present the average chirp period of the 2010 data as Wildtype (WT)

population. However, these recordings were too short (10-20 chirps per animal) to calculate representative chirp period C.V.

Detection of continuous calling

Across all recordings used in this study, chirp durations were overwhelmingly shorter than 3s. For the Florida population 149 chirps were longer than 5s. These 149 chirps covered 18.2% of the total calling time analyzed for the Florida population (17,817 chirps in total), so they represent a significant part of this population's calling behavior. We excluded these 'calling pieces' with durations longer than 5s from the chirp analysis and analyzed them separately, i.e., we present the fraction of males producing such long calling pieces and the fraction of their calling time covered by these long call pieces in separate analyses. In contrast, only one of all recorded Puerto Rico males produced a single calling piece that was longer than 5s (out of 14,052 chirps analyzed).

Statistics

We reared animals from hatching to adult molt in long-day conditions (Long-day control), short-day conditions (Short-day control), and collected animals from the field in Florida and Puerto Rico (Wildtype or 'WT') as references. Note that we did not record animals collected as adults in Puerto Rico for this study and used recordings that were done in the context of another project (Beckers and Schul, 2010). We used these recordings to determine the DP-rate and chirp period but not for the C.V. of the pulse period because we did not have long enough recordings to calculate a C.V. that would truly represent the consistency of the chirp pattern. We compared the double-pulse rate and chirp period in two separate ANOVAs for each population. One ANOVA compared the double-pulse rate across the controls (Long-day, Short-day, WT) and another ANOVA those of the rearing treatments within each population (0 days to 140 days). We used post-hoc Tukey tests to determine significant differences between control groups and treatment groups within each population. We compared the double-pulse rates and chirp periods between Florida and Puerto Rico WT animals using t-tests. We calculated for each recording the C.V. of the chirp period to compare the variability as a measure for chirp pattern quality. We used ANOVAs and post-hoc Tukey tests to compare the C.V.s across treatments within each population.

In contrast to Florida animals, only one of all Puerto Rico animals produced a long call piece (i.e., > 5s). To compare the proportion of males producing calling pieces longer than 5s in the Florida population, we used a Fisher exact test. Because very few Florida animals produced these calling pieces in the three control groups and 28-day treatment (N = 2 - 4), we could not run an ANOVA across the data to test the proportion of calling that consisted of such long calling pieces. To test for a change in this proportion toward longer treatment durations, we pooled the data of the 56- and 84- day treatments and compared those data to that of the pooled 112- and 140-days treatments using a t-test. All data

analyzed with parametric tests complied with normality and homogeneity of variance assumptions of these models. We used JMP (version 16.2.0 for Mac) for all statistical analyses.

Results

Double-pulse rate

The DP-rate of wild-collected Florida animals did not differ from that of Puerto Rico animals (T-test, t-ratio = 1.544, p = 0.146). Next, we compared the DP-rates among the three controls (Wildtype, Long-day, Short-day) within each population to test for effects of juvenile development on this call trait. DP-rates for the Puerto Rico population were around 105 DP/s for all three controls (Figure 2A) and the DP-rates did not differ among controls (ANOVA, F-ratio = 1.15, p = 0.33). In the Florida population (Figure 2B), Wildtype controls had an average DP-rate of 104.8 DP/s and those of Long-and Short-day controls were 98.7 and 91.3 DP/s, respectively. These controls differed significantly (ANOVA, F-ratio = 16.70, p < 0.0001) and *post-hoc* tests revealed significant differences among all three controls (all *post-hoc* Tukey tests: p < 0.02).

Next, we tested for effects of diapause duration on DP-rate in each population. These treatments had the same juvenile developmental conditions as the Short-day control (= 0-day diapause) and differed in the duration that the animals stayed as adults in diapause conditions. DP-rates of Puerto Rico males differed significantly across diapause durations (ANOVA, F-ratio = 24.92, p < 0.0001), decreasing from about 105 DP/s at short diapause durations to 81.2 DP/s at 138 days (Figure 2A). DP-rates of animals that spent 15 to 52 days in diapause did not differ from the Short-day control (= 0 days; all *post-hoc* Tukey tests: p ≥ 0.99). Animals that spent more than 100 days in diapause had significantly slower DP-rates than the Short-day control (all *post-hoc* Tukey tests: p < 0.0001). DP-rates at 123 and 138 days were significantly lower than those of all groups up to 95 days (all *post-hoc* Tukey tests: p ≤ 0.0004).

In the Florida population, the DP-rates of diapause treatments ranged from 83.5 to 91.3 DP/s (Figure 2B) and even though our analysis detected a significant difference (ANOVA, F-ratio: 3.12, p = 0.014) none of the treatments differed significantly from the Short-day control (= 0-days diapause) or among treatments (all *post-hoc* Tukey tests: p ≥ 0.051). Notably, even though marginally not significant (0.051 ≥ p ≥ 0.073), the 84- to 140-day diapause treatments resulted in the slowest DP-rates compared to the Short-day control, possibly indicating a further reduction in DP-rate due to diapause.

Chirp pattern

The characteristic chirp pattern of *N. triops* was affected during diapause and continuous calls were typically only observed in the 'winter' generation (Whitesell and Walker, 1978; Beckers and Schul, 2008). Examples of the time course of chirp periods within calling

bouts are shown in Figure 3. Most males from Puerto Rico showed little variation of the chirp period during each calling bout while some males produced shorter chirp periods at the beginning and end of each calling bout (Figure 3A). These patterns appeared in all treatment groups and were not correlated with rearing or diapause conditions.

Males from Florida showed similar chirp period patterns in all treatment groups, however, chirp periods were typically more variable than those of the Puerto Rico population (Figure 3B and see below). In addition, a distinctly different pattern occurred in all Florida treatment groups, i.e., calling bouts started with a distinct chirp pattern and lengthening chirp periods over 10-90s before switching to periods of 'continuous calling' (i.e., call pieces longer than 5s) of variable duration (range 5.5s – 489s) followed by a short stretch of chirps at the end (Figure 3C). Almost all cases of continuous calling followed this temporal chirp pattern in the Florida population. We also observed 'transitionary' patterns that never reached call pieces longer than 5s (Figure 3C, right). Only one male of the Puerto Rico population (N = 57) produced a single call piece longer than 5s. The time course of chirp periods during this calling bout was comparable to Florida calls (Figure 3D).

To quantify the effect of juvenile development and diapause on the chirp pattern, we analyzed chirped and continuous call sections separately. Comparing the chirp periods of Wildtype animals between Florida and Puerto Rico populations indicated that the chirp periods of Florida animals were significantly longer than those of the Puerto Rican animals (T-test, T-ratio = -4.83, p = 0.0002). The average chirp periods of Puerto Rico males ranged between 980 and 1032ms for the three control treatments and they did not differ significantly from each other (ANOVA, F-ratio = 0.59, p = 0.56; Figure 4A). Chirp periods differed significantly across diapause treatments (ANOVA, F-ratio = 3.54, p = 0.005). However, the only significant differences were that the 30-day chirp period was shorter than those of the 61-, 123-, and 138-day treatments (all *post-hoc* Tukey tests: p ≤ 0.026), while none differed from the Short-day control (*post-hoc* Tukey tests, all p ≥ 0.17).

Mean chirp periods of Florida males of the three control treatments ranged between 1239.5 and 1356.9ms and did not differ significantly (ANOVA, F-ratio= 1.49, p = 0.239; Figure 4A). Mean chirp periods increased from the Short-day control with increasing diapause duration to 1692ms at 140-days of diapause, however, this increase was not significant (ANOVA, F-ratio = 1.90, p = 0.109; Figure 4A).

We use the coefficient of variation (C.V.) of individual males' chirp periods to evaluate the within-male consistency of the chirp rhythm (Figure 4B). In the Puerto Rico population, we detected no difference between the Long-day and Short-day control (= 0-day, T-test, t-ratio = -0.83, p = 0.424). In the Florida population, we detected a significant difference across the three controls (ANOVA, F-ratio = 5.59, p = 0.008), with the C.V. of Short-day controls being larger than those of Wildtype animals (*post-hoc* Tukey test, p = 0.006). In the Puerto Rico population, our analysis detected a significant effect of diapause treatment on the chirp period C.V. (ANOVA, F-ratio = 2.40, p = 0.041), yet *post-hoc* comparisons did not identify significant differences between any two treatments (all *post-hoc* Tukey tests, p ≥ 0.11). Similarly, the mean C.V. of the
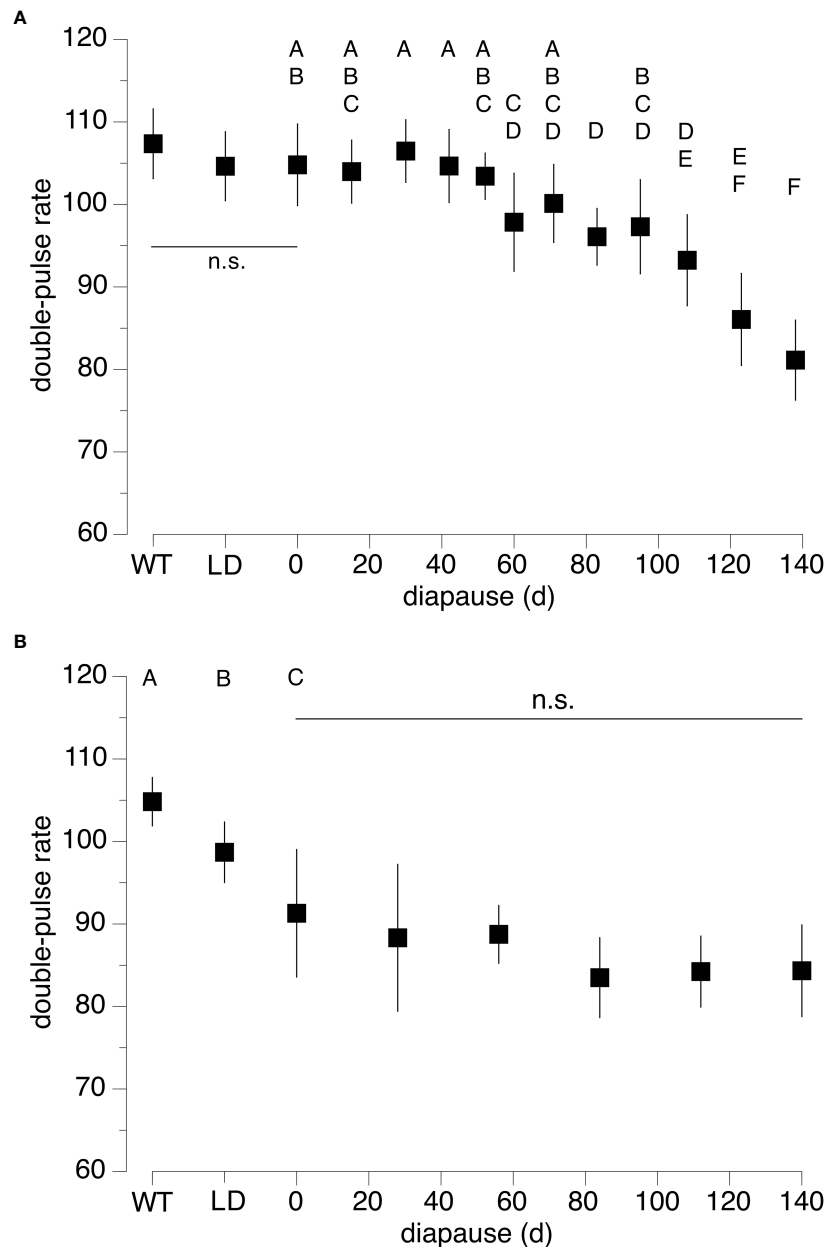
FIGURE 2
Double-pulse rate (mean ± SD) of Puerto Rico **(A)** and Florida **(B)** *N. triops* in response of rearing conditions. Wildtype (WT) males were collected in the field, Long-Day controls (LD) had juvenile development and post adult molt maturation in 15:9 light/dark cycle and did not experience diapause conditions. All other treatment groups had juvenile development with 11/13h light/dark cycle and diapause conditions of 9.5/14.5 light/dark for the indicated durations. The 0-day diapause treatment corresponds to the Short-Day control. 'n.s.' indicates no significant statistical difference. Different letters between treatments indicate significant differences (p < 0.05).

Florida diapause treatments did not differ significantly across diapause durations (ANOVA, F-ratio = 0.77, p = 0.58).

The proportion of Florida males producing long call pieces (> 5s duration) did not differ among the Wildtype, Long-, and Short-day controls (Fisher Exact Test: p = 0.501; Figure 5A). However, the proportion of males producing long calling pieces significantly increased with diapause duration from about 20% at 0 days and 28 days of diapause to 70% at 140 days of diapause (Fisher Exact test: p = 0.049). The proportion of total calling time covered by long (> 5s) calling pieces was highly variable among males (Figure 5B).

The proportion of long calling pieces increased significantly between intermediate treatment durations (pooled 56- and 84-days) compared to long treatment durations (pooled 112- and 140-days; T-test, t-ratio = 2.35, p = 0.031).

Discussion

Phenotypic plasticity of DP-rate in *N. triops* was a quantitative trait in both tested populations (Figure 2). Induction of the alternate
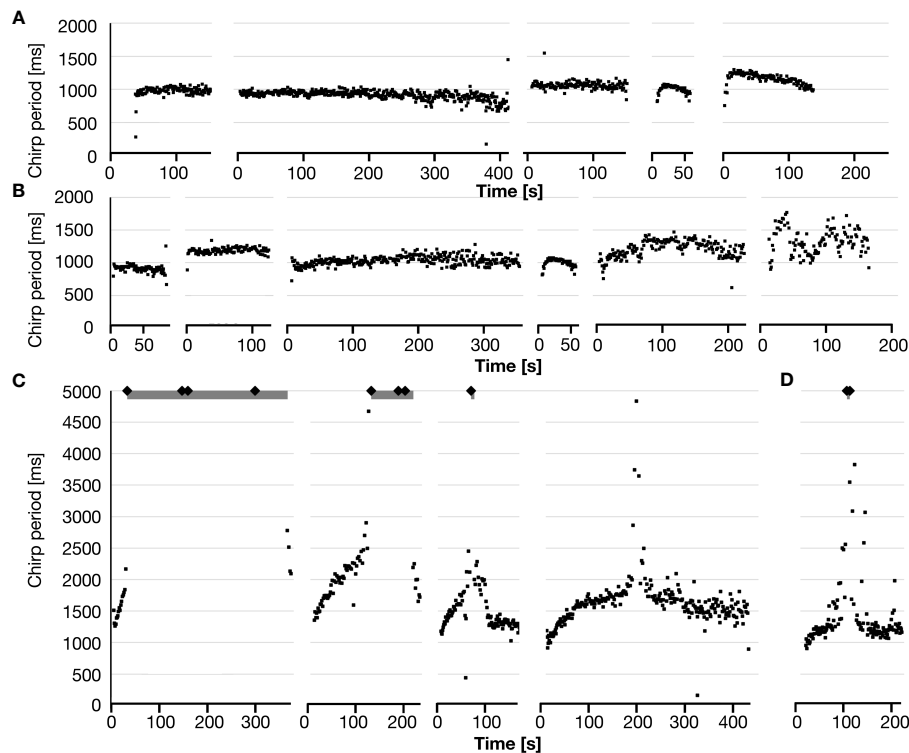
FIGURE 3
Examples for the time course of chirp periods during a calling bout of male *N. triops*. The chirp periods are plotted at the time when they occurred during a calling bout. **(A)** Five calling bouts of Puerto Rico males without call pieces > 5s. Each calling bout was produced by a different male. **(B)** Six calling bouts of Florida males without call pieces > 5s. Each calling bout was produced by a different male. **(C)** Four calling bouts of Florida males with longer call pieces. The gray bars at the top indicate times with continuous calling (i.e., calling pieces longer than 5s) and the diamonds indicate the beginning of such long call pieces. The rightmost calling bout fell just short of reaching the 5s threshold. Each calling bout stems from a different male. **(D)** The only calling bout of a Puerto Rico male with a call piece >5s (Long-day control male). Note that the timescale in **(C, D)** are different from **(A, B)**.

(=low) pulse rate occurred at different developmental stages, i.e., juvenile stage in Florida animals (Figure 2B) and adult diapause in Puerto Rico animals (Figure 2A). Unexpectedly, phenotypic plasticity of chirp structure, which occurred almost exclusively in the Florida population, was also a quantitative trait (Figure 5B). In Florida males, the expression of the alternative continuous call structure became more prominent with longer duration of adult diapause (Figure 5B) and took place after the induction of the alternative double-pulse rate. These results support our prediction that phenotypic plasticity of double-pulse rate and chirp structure are both independently induced and likely independently evolving traits.

Temperate winter and evolution of diapause

When *N. triops* expanded its range into temperate North America, it experienced environmental conditions during winter that interfered with its tropical life history: Offspring of the summer generation maturing in late fall were exposed to temperatures too cold for reproduction. Thus, this population experienced strong selection for adult diapause, i.e., arresting development after the fall adult molt and delaying reproduction until spring. Observed

differences in life history between Florida and Puerto Rico populations seem to support this selection hypothesis for adult diapause: Independent of diapause duration, short-day raised Florida males did not start calling, unless exposed to long-day conditions for more than four weeks, suggesting that significant maturation steps were still required for calling. We have observed this same pattern in previous studies as well (Beckers and Schul, 2008; Beckers and Schul, 2010). In contrast, Puerto Rico Males raised in the same conditions started calling two to three weeks after adult molt, even when remaining in short-day conditions (J.S. personal observation). Puerto Rico males occasionally called while in diapause and, when transferred to long-day conditions after diapause, most males called within one week. Thus, development of Puerto Rico males was not arrested and their inactivity in diapause conditions was likely caused by the low temperatures (12-17°C).

The arrested development induced during juvenile development in the Florida population exceeded the capacity of compensatory (or homeostatic) mechanisms that otherwise maintain the standard call phenotype (i.e., fast pulse rate, chirped call structure) in the more stable environment of the tropics. The resulting alternative call phenotype represents a reversal to an ancestral state in call structure (i.e., continuous calling for *Neoconocephalus;* Frederick and Schul, 2016), as well as a substantial change in a critical call parameter for female call recognition (i.e., double-pulse rate; Beckers

and Schul, 2008). Neither of these call changes seem to be adaptive, in terms of resulting in an increase in attractiveness to females. Rather to the contrary, the alternate, slower double-pulse rate reduces female attraction and affects males' fitness (Beckers and Schul, 2008). Thus, the increased diversity in mating calls expressed in the 'winter' generation of *N. triops* is likely a byproduct of selection for diapause rather than the result of direct selection on these traits. We discuss below first the evolutionary context of this call plasticity and second its neural basis.

Call variation as the result of phenotypic plasticity

Initially, phenotypic plasticity of *N. triops* was described as two categorical call phenotypes: A summer (or standard) call and a winter call (or alternative) call (Whitesell, 1974; Whitesell and Walker, 1978). The standard call is rhythmically interrupted into chirps and has a ~20% faster double-pulse rate than the slower and continuous alternate call. However, our results indicate that this categorization needs to be revised. First, the double-pulse rate and the call structure are not categorical but quantitative traits. Second, population differences as well as large scale differences in the timing of induction of either call trait indicate that the call is modular, i.e., the two call traits can be induced independently of each other. Note that in Florida the change in DP-rate was induced during juvenile development, whereas in Puerto Rico it changed during adult diapause. We report here a previously undescribed combination of call structure and pulse rate resulting in a new call phenotype: Puerto Rican males that were kept in diapause for long periods produced a chirped call (standard structure) with the slow DP-rate of the alternate call (Figure 2A: >100d; none of these animals produced call pieces longer than 5s). Similarly, Florida males that we reared in short-day conditions as juveniles until adulthood or additionally kept for 28 days in diapause afterwards produced chirped calls with slow pulse rates (Figures 2B, 5A: 0-28d; slow DP-rate but majority of males called with chirps). The quantitative and modular nature of call plasticity ultimately contributes to even more phenotypic variation in this calling behavior than initially assumed, because intermediate states of both call traits and new combinations can be expressed as well.

When *N. triops* expanded its range into temperate North America, male call plasticity challenged the match between signals and preferences in the new environment, requiring female preferences to evolve in response to male call changes (Beckers and Schul, 2010). The developmental capacity to express quantitative, smaller incremental changes rather than a few large qualitative changes in the mating calls may have facilitated the necessary co-evolution between calls and female preferences (Beckers and Schul, 2010). Thus, female preference followed rapid phenotypic change of male calls here, making this system another example that challenges the sexual selection paradigm that male signals typically evolve in response to female preferences (Bush and Schul, 2010).

Phenotypic plasticity can be a potent mechanism to provide phenotypic variation in species-specific traits (e.g., pulse rate) necessary to propel the evolution of communication systems that female mate recognition typically keeps rather invariable (Gerhardt

and Huber, 2002). Our experimental design allowed us to detect intermediate trait states and modularity of the variable call traits, displaying the potential of novel behavioral phenotypes in *N. triops*. It is possible that a similar approach in other plastic communication systems may reveal further signal variation than has been described (e.g., *Gryllus rubens*: Walker, 2000; Beckers et al., 2019; *Laupala cerasina*: Grace and Shaw, 2004; *Allonemobius fasciatus*: Olvido and Mousseau, 1995). The trait variation of other behavioral and morphological traits that have been described largely as categorical (e.g., reproductive alternatives: Eberhard and Gutiérrez, 1991; Rowland and Emlen, 2009; feeding alternatives: Collins and Cheek, 1983; Pfennig, 1992; seasonal alternatives: Shapiro, 1976) might merely represent ends of a quantitative trait spectrum and may benefit from being tested, analyzed, and redescribed in a more nuanced way. Considering the complexity of phenotype induction through multiple environmental factors acting at various time points (review in West-Eberhardt, 2003), it would not be surprising if phenotypic plasticity is generally more quantitative in nature with graded phenotypes induced over a range of stimuli rather than two or few categorical phenotypes induced at discreet thresholds.

Evolution of call plasticity and evolutionary ramifications

In the temperate environment, adult diapause in *N. triops* is crucial for survival through the winter. Thus, selection should favor a lower threshold and/or earlier induction of the physiological changes for diapausing. In contrast, in tropical environments where *N. triops* breeds all year long (Walker and Greenfield, 1983), induction of diapause would be maladaptive by prolonging generation time even though conditions would allow breeding. Note that daylength, i.e., the environmental factor inducing diapause in Florida, is more constant in Puerto Rico than in Florida, it still varies significantly throughout the year. Selection therefore should favor higher thresholds for diapause induction in this population, leading to divergence of the threshold value between Florida and Puerto Rico populations. The drastically later induction of DP-rate changes (juvenile vs. adult stage) and lack of call structure changes in the Puerto Rico population might indicate such a divergence in threshold value between populations. Similarly, the threshold for minor and major male morphs in the polyphenic dung beetle *Onthophagus taurus* has evolved among populations, likely as the result of regional differences in inter- and intraspecific competition (Moczek, 2003). These studies highlight how phenotypic plasticity can contribute variation necessary for selection to act and populations to evolve.

Consistent and repeatable environmental conditions can lead to the permanent expression of only one, more suitable phenotype in a previously plastic system (West-Eberhardt, 2003). In this case, selection through genetic accommodation can act to genetically fix this phenotype (genetic canalization; Waddington, 1961). In essence, the threshold evolves to be not responsive to environmental input any longer, resulting in the loss of plasticity (West-Eberhardt, 2003). This evolutionary scenario could play out in *N. triops* in more seasonal temperate regions further north from Florida where the reproductive season is significantly shortened and does not
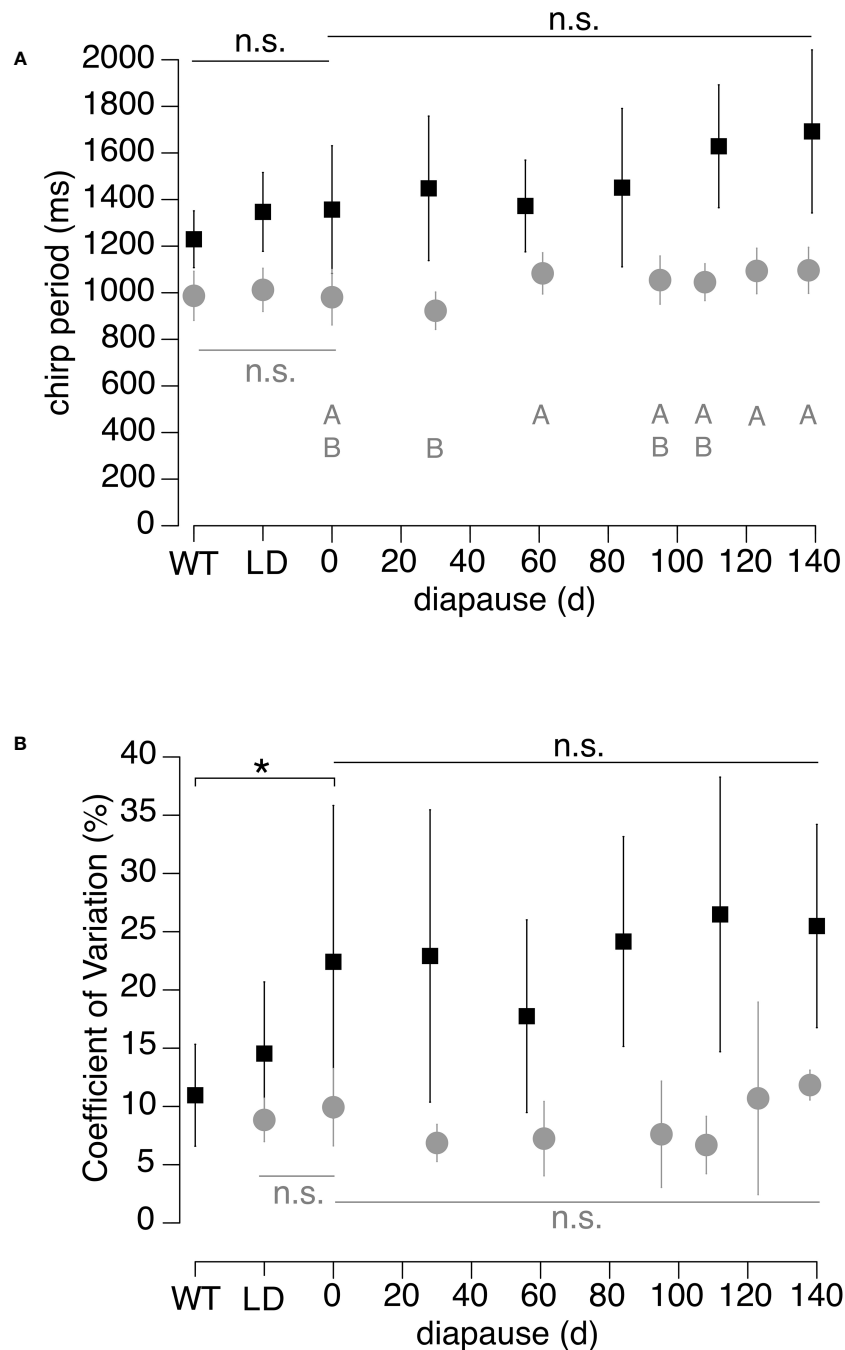
FIGURE 4
(A) Chirp period duration (mean ± SD) of Florida (black symbols) and Puerto Rico (grey symbols) *N. triops* at different rearing treatments. **(B)** Mean of within-male Coefficient of Variation (C.V.) of the chirp period of the measurements shown in **(A)**. The C.V. is used as a proxy for the "quality" of the chirp rhythm. WT males were collected in the field, Long-day (LD) males with juvenile development and post adult molt maturation in 15/9h light/dark cycle and no diapause. All other males developed as juveniles in a 11/13h light/dark cycle and diapaused in a 9.5/14.5h light/dark cycle. The duration in diapause is indicated on X-axis. The 0-day diapause treatment corresponds to the Short-Day control. 'n.s.' indicates no significant statistical difference. Different letters and asterisk indicate significant differences between treatments (all p < 0.05).

allow for a second generation to occur in late summer (Whitesell, 1974). These conditions are met at the northern edge of *N. triops'* range where the katydid has only one reproductive generation per year and all individuals diapause as adult until the following spring (OMB personal observations; SINA, 2023). We are currently testing the hypothesis that this northern population of *N. triops* has lost its capacity to express both call phenotypes.

Given that the alternative (= slower) double-pulse rate appears to be a pleitropic effect of adult diapause induction (Beckers and Schul, 2010), canalization of diapause should also genetically fix this alternative call trait. Further, this should select in these univoltine populations for female preference for the lower pulse rate. Canalization of the alternative call phenotype could potentially lead to reproductive isolation between this temperate univoltine
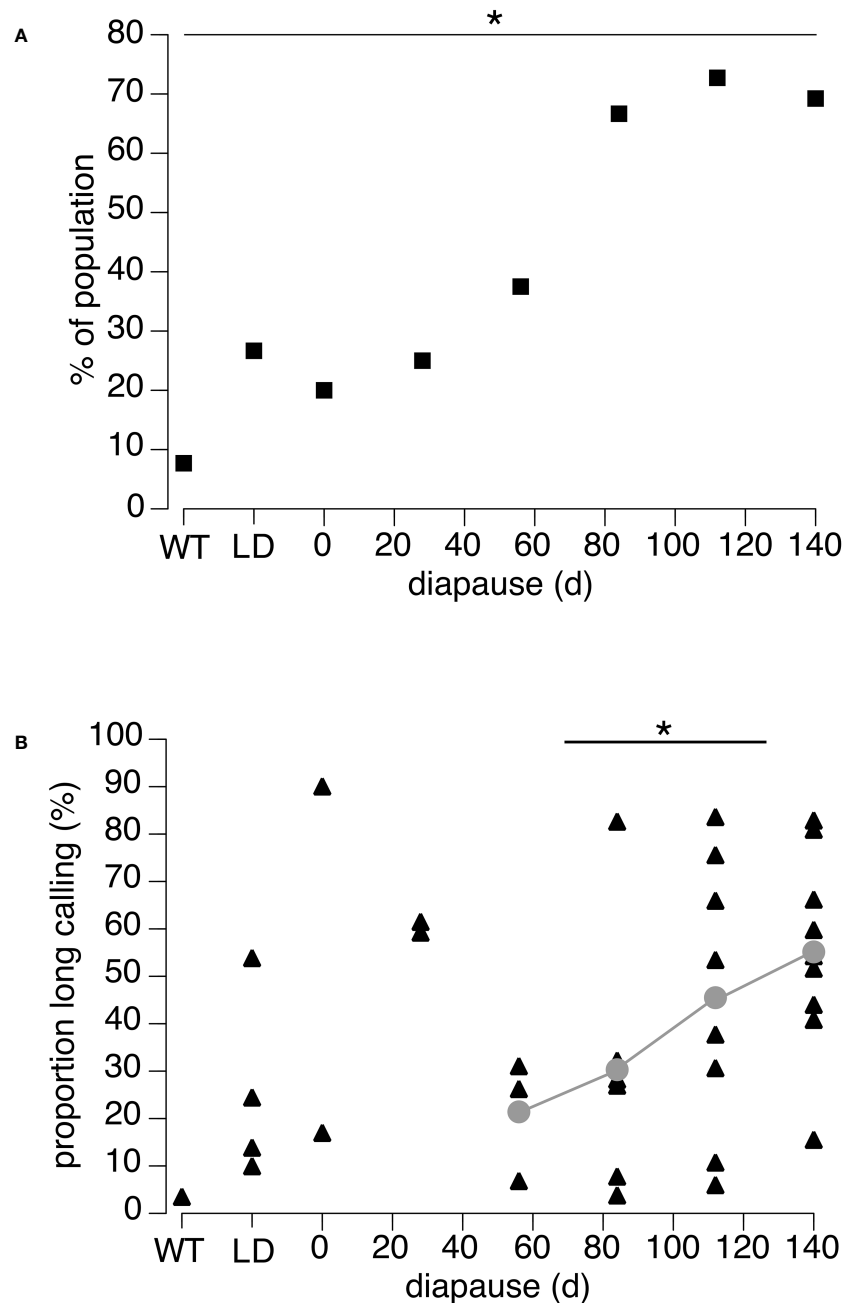
FIGURE 5
Continuous calling of the Florida population of *N. triops*. **(A)** Proportion of males of each treatment that produced call pieces longer than 5s (N = 8-15 per treatment). **(B)** Proportion of long calling pieces (>5s chirps) relative to the total calling time. Individual data points and means (grey) for treatments 60 days and longer are shown. The grey trendline connects only the data used in the analysis (see Methods). Note that out of all analyzed animals (N = 8-15 per treatment) only those are shown that produced long calling pieces. Each symbol represents data from one animal. WT males were collected in the field, Long-day (LD) males with juvenile development and post adult molt maturation in 15/9h light/dark cycle and no diapause. All other males developed as juveniles in a 11/13h light/dark cycle and diapaused in a 9.5/14.5h light/dark cycle. The duration in diapause is indicated on X-axis. The 0-day diapause treatment corresponds to the Short-Day control. Asterisks indicate significant differences (p < 0.05).

population and tropical *N. triops*, depending on the location of the secondary contact. The bivoltine Florida population expressing both call types currently acts as a bridge for gene flow between the northern and tropical populations.

The two populations of *N. triops* also differed substantially in plasticity of their call structure, with one population displaying different call structures (Florida), whereas the other population seemingly did not (Puerto Rico). In the Florida population, continuous callers occurred in all treatment groups and their proportion reached ~70% after three months in diapause. In contrast, only a single Puerto Rico male produced a single continuous call section across all treatment groups. Female preferences for call structure shows striking geographic variation (Beckers, 2008). Females from Costa Rica require chirped calls and

do not show significant responses to continuous calls. In contrast, females from Florida respond equally well to chirped and continuous calls (Beckers and Schul, 2008). The loss of selectivity for chirped calls in the Florida population may be a consequence of the diapause-related continuous calls in the winter generation, i.e., the loss of chirp preference facilitated successful communication of winter animals. Interestingly, females from Puerto Rico also lack the preference for chirped calls and respond to chirped and continuous calls equally well (Beckers, 2008). This pattern has two possible explanations. First, the loss of preference for chirped calls evolved in Florida as a response to the continuous calls of the winter generation and spread to Puerto Rico. Alternatively, the loss of preference may have occurred in tropical populations and facilitated the spread into temperate Florida by mitigating the effect of the plasticity of the call structure. Existing data does not allow to distinguish between these hypotheses.

Neural basis of call plasticity

Generation of the double-pulse pattern requires some form of neural resonance in the central call pattern generator (Marder, 2000). Such resonances may occur within single neurons, i.e., across cell membranes, or among neurons or networks involving a combination of membrane and synaptic properties (Hutcheon and Yarom, 2000; Izhikevich, 2001). Tuning of resonances relies typically on ratios of currents rather than absolute values. Thus, similar outcomes can be achieved with different combinations of current levels (Schulz et al., 2007; Franklin et al., 2010; Hudson and Prinz, 2010). Changing of the excitation/inhibition balance within the network is another potential mechanism to tune central pattern generator resonances (Buzsáki and Wang, 2012). Importantly, changing neural resonance likely is the outcome of quantitative changes of gene expression and does not require major re-organizations of the neural topography of the central pattern generators.

In Florida *N. triops*, the alternate double-pulse rate was induced during juvenile development. Here, the environmental conditions affected the tuning of the double-pulse pattern generator before it became functional after adult molt. In the Puerto Rico population this change occurred after the adult molt, i.e., after experiencing short-day conditions and extended periods of diapause conditions. At the time of the change, the double-pulse central pattern generator was likely already functional, which was indicated by calling of some Puerto Rico males in short-day and diapause conditions. Thus, in Puerto Rico the change in double-pulse rate was likely the result of changes in an already existing resonance rather than the development of a slower resonance from the start. Determining which neural changes underlie the changes of double-pulse rate in the two populations would require more detailed neural and transcriptomic studies.

Crickets and katydids have separate CPGs for chirp and pulse pattern which are located in different ganglia in the thoracic/abdominal central nervous system (Schöneich and Hedwig, 2011; Jacob and Hedwig, 2016; Katydids: JS in prep.). Males of the Florida population that produce continuous call pieces also produce chirped calling before and after continuous calling parts (Figure 3), indicating that the chirp generator is functional. The integration of the chirp pattern with the pulse rhythm, however, seemingly breaks down during continuous calling. Thus, time spent in diapause likely interferes with the integration of the two patterns during the post adult maturation of the central nervous system. Similarly, the increased chirp period C.V. of diapausing Florida males (Figure 4B) suggests that induction of diapause also deteriorated the quality of the chirp pattern in addition to the effect on pattern integration.

Conclusion

Climate change, colonizations, and invasions can expose species to novel or more extreme environmental conditions (Hughes, 2000; Ghalambor et al., 2007). In these instances, homeostatic mechanisms that buffer typical environmental variation are challenged (Rutherford, 2000), exposing novel trait variation through phenotypic plasticity (Ghalambor et al., 2007). The exposed hidden genetic variation underlying these novel traits may then become a necessity for species to evolve (Rutherford, 2000) and successfully settle or survive in these new environmental conditions. Thus, hidden genetic variation exposed to selection through plastic phenotypes may represent the basis for some (pre-)adaptations and may determine the ability of individuals to cope with environmental challenges beyond the natural range of variation (Rutherford and Lindquist, 1998; Ghalambor et al., 2007). The value of studying invasive species experiencing novel environmental conditions lies in better understanding and predicting possible evolutionary responses to climate change, which has recently gained attention (reviews in Caplat et al., 2013; Moran and Alexander, 2014). Our study provided important insights into these processes in the context of sexual communication, a suite of traits that aids in species isolation but also speciation (e.g., Arnegard et al., 2010). Further research into the hidden genetic capacity is of importance to our understanding of species existence, extinction, and evolution in face of rapidly changing environments.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The manuscript presents research on animals that do not require ethical approval for their study.

Author contributions

Acknowledgments

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2023.1216463/full#supplementary-material

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