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RESEARCH ARTICLE

# Hatching Date Variability in Wild Populations of Four Coexisting Species of African Annual Fishes

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**Background:** Hatching is modulated by a combination of intrinsic and extrinsic factors. Annual killifish are adapted to complete their entire life cycle in annually desiccating habitats. Spending most of their life in the embryonic stage, they have evolved adaptations to survive desiccated conditions and match their hatching with the unpredictable onset of the aquatic phase of the pool. We examined spatial and temporal synchrony of hatching in natural populations of four species of African annual killifish (genus *Nothobranchius*). We compared differences and variability in hatching dates among years, regions, pools, and species and matched them with data on inundations of individual pools. **Results:** Inundations typically coincided with peak rainfall in early January. We found considerable spatial and temporal synchrony in 1 year, but less synchrony in the other 2 years. Hatching generally occurred 0–20 days after inundation; fish at most sites hatched synchronously (<1 week) but some sites showed protracted hatching or two age cohorts. One species tended to hatch earlier than the other three. **Conclusions:** We suggest that hatching of annual killifish in the wild is a result of the interplay between environmental conditions and individual predisposition to respond to threshold environmental cues, ensuring effective bet-hedging against unpredictable inundation. *Developmental Dynamics 246:827–837, 2017*. © 2017 Wiley Periodicals, Inc.

**Key words:** developmental synchrony; diapause; *Nothobranchius*

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## Introduction

The transition between embryonic and postembryonic life is a major salient shift in ontogenetic development. In egg-laying animals, it requires hatching from the egg envelope and transition to life in an external environment. Hatching is initiated by extrinsic and intrinsic cues and is typically obligatory within a short timespan once a defined developmental threshold is reached. The rate of embryo development, and hence the timing of hatching, can be affected by parental care and modulation of the incubation environment, such as exposure to lower or higher temperatures (Spencer et al., 2001). For example, birds lay a single egg a day but some species start to incubate the entire clutch only after all the eggs are laid, synchronizing embryo development among individuals (Hillström and Olsson, 1994). The developmental rate of embryos that are not actively incubated by parents can be manipulated through deposition in thermally optimal microhabitats (Dvořák and Gvoždík, 2010). Furthermore, intrinsic factors (genetic or maternal) can modulate the speed of embryo development (Rafferty and Reina, 2012), and variation in embryo development rate has been reported between closely related species (Beacham and Murray, 1990) and among individuals within species (Williams, 1994).

Annual killifishes are renowned for their ability to match hatching with suitable environmental conditions. This is a crucial adaptation, as this group of freshwater fishes inhabits regularly desiccating habitats (Reichard, 2015; Furness, 2016). The populations persist by depositing eggs that can withstand habitat desiccation. The eggs are buried in the pool sediment and start their development in an aquatic environment. After habitat desiccation, the eggs remain encased in the dry mud until the next inundation that comes after several weeks, months, or even years (Polačik et al., 2014; Cellerino et al., 2016). Adaptations to cope with such abrupt changes in developmental conditions include a thickened chorion with surface filaments (Podrabsky et al., 2001) and a series of three facultative diapauses where embryo metabolic rate is reduced to approximately 10–15% and development is arrested (Podrabsky and Hand, 1999; Furness et al., 2015a).

African annual killifishes from the genus *Nothobranchius* belong to one of six independent clades of the order Cyprinodontiformes where an annual reproductive mode has been recorded (Furness et al., 2015a). While laboratory studies on the rate and flexibility of annual killifish embryo development have received considerable interest, nothing is known about their developmental trajectories in the natural environment. Laboratory studies have revealed that embryo development, and its propensity to enter any of the three developmental diapauses, is modulated by ambient temperature, photoperiod, chemical cues from adult fish

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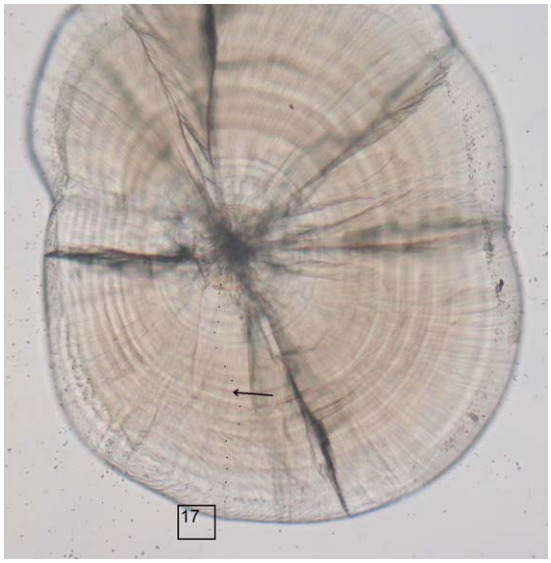


**Fig. 1.** An example of otolith section, with daily increments apparent as the alternation of dark and bright circuli (indicated by black dots and arrow). *Nothobranchius pienaari* collected in the wild, Limpopo South region, 17 days old. Original magnification, 100×.

and oxygen availability (Wourms, 1972a,b,c; Inglima et al., 1981; Levels and Denucé, 1988; Polačik et al., 2014, 2017; Furness et al., 2015b).

Investigations of the natural environment of African annual killifishes suggest that embryos are likely exposed to the anoxic sediment of the pool bottom, followed by a dramatic shift to desiccated conditions once water evaporates from the pool, subsequent wetting of the sediment from initial rains, and complete inundation after the main seasonal precipitation (Watters, 2009; Reichard et al., 2015). It has been speculated that embryos may spend the periods of the harsh environmental conditions (anoxia, dryness) in one of three facultative diapauses (Wourms, 1972c; Furness, 2016) and advance their development in short transient periods when oxygen and water are available (Watters, 2009; Reichard, 2015). However, laboratory studies suggest a major contribution of genetic (Furness et al., 2015a; Vrtílek et al., this issue) and maternally transmitted epigenetic (Polačik et al., 2017) effects on embryo development trajectories. The relative contribution of environmental and intrinsic factors to the natural duration of embryo development culminating in hatching and transition to postembryonic life is unknown.

In the present study, we examined spatial and temporal synchrony of hatching in natural populations of four species of African annual killifish from a particularly dry part of their geographic range. We back-calculated individual hatching dates from age estimates of individuals collected in the wild as juveniles and adults and compared differences and variability in hatching dates among years, regions, pools, and species. We correlated individual age estimates with body size to test whether size is a relevant predictor of the age. We matched estimates of hatching dates with data on pool inundations obtained from data loggers deployed in the pools to analyze the timing of hatching with respect to habitat inundation.

The study was conducted in southern and central Mozambique, across the entire range of the Southern clade of *Nothobranchius* (Dorn et al., 2011, 2014; Bartáková et al., 2015). Over this large area, three species complexes coexist in sympatry and often co-occur locally, while species within each complex are always allopatric (Reichard et al., 2017). The ranges of all species are structured into geographically well-defined regional clades (Bartáková et al., 2013, 2015). Five main regions were considered in the present study. Over 3 years of sampling (2010, 2012, 2013), we collected juvenile and adult fish from wild populations and estimated their date of hatching by counting the daily increments deposited in the otoliths, mineralized structures of the inner ear (Fig. 1; Polačik et al., 2011; Reichenbacher and Reichard, 2014). We targeted as large a geographic area as possible (Fig. 2) and collected fish from a wide range of habitat types. Sample size depended on the size structure of adult fish and the number of species in the pool. We analyzed larger samples from sites where fish size variability was high, multiple size cohorts were present, or several species coexisted. We additionally deployed temperature loggers at various sites across the study area (Fig. 2) and estimated the dates of habitat inundation from abrupt changes in daily temperature fluctuations (Terzibasi Tozzini et al., 2013; Blažek et al., 2017).

We tested the following predictions, based on information from a pilot study by Polačik et al. (2011). First, we predicted that hatching dates would be consistent across years, associated with peak seasonal precipitation in early January. Second, we predicted that hatching would be synchronous across the study area and within particular regions and individual pools, and there are no inter-specific differences in hatching dates. Third, we predicted the occasional occurrence of multiple generations of fish within the same rainy season, especially in the driest region where precipitation is erratic.

## Results

### Inter-annual and Regional Variability

In 2012, hatching was relatively synchronous across the entire study area except the Beira (northernmost) region. In 2010 and 2013, hatching was considerably less synchronous (Table 1; Fig. 3). Quantitatively, this is manifested by a direct comparison of standard deviations (SD) in mean hatching dates, with markedly lower SDs in 2012 (Table 1). Variance components analysis revealed that variability among years explained 35.7% of total variation, followed by variability among the regions (22.0%) and variability among the populations within the regions (19.1%).

Mean hatching date was earliest in 2012 (26 January), compared with 15 February in 2010 and 17 February in 2013 (GLM: $F_{2,12.7} = 11.3$; $P = 0.002$). The same pattern held for all four killifish species when compared separately (Table 1). There was no consistent pattern across years to suggest that hatching dates were regularly earlier in a particular region (Fig. 3).

### Age Cohorts Within a Pool

There was clear evidence of multiple age cohorts within a single year in some pools, although their occurrence was rare. Typically, hatching of each cohort appeared to continue for several days at each site. In a subsample of sites with at least three valid otolith readings (58 sites/years), 41% showed all fish hatched within a period less than 10 days, while 40% had a range of 10–20 days between the first and last fish hatching (Fig. 4). It is important to note that the magnitude of protracted hatching and the evidence

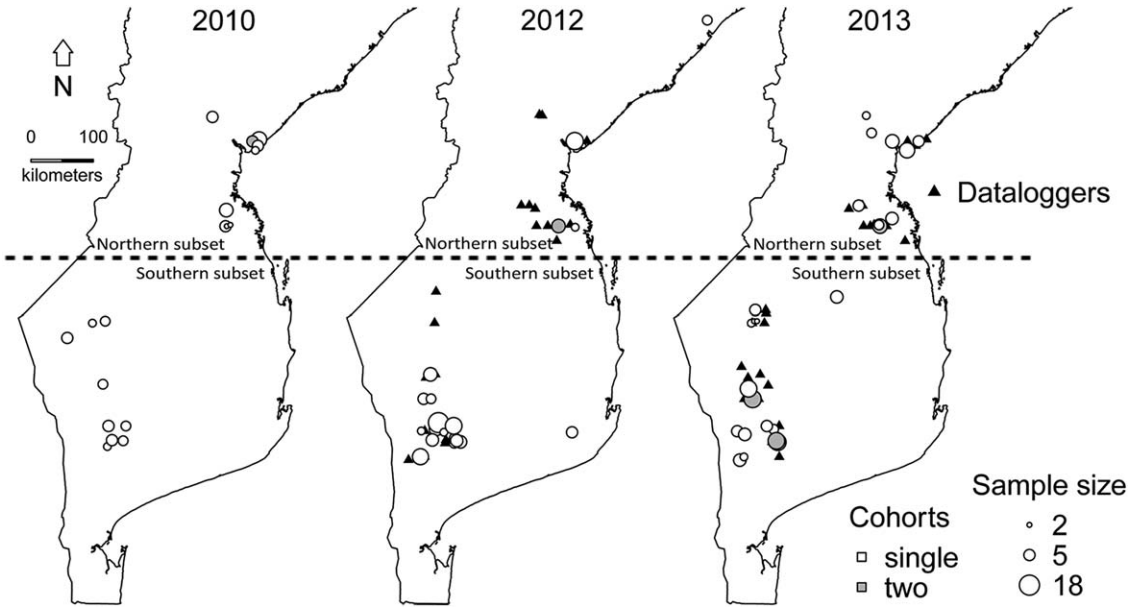


**Fig. 2.** Map of the study area showing sites where the hatching dates were estimated from otoliths (circles) and inundation time was successfully read from dataloggers (black triangles). Shaded circles denote multiple age cohorts per year and open circles a single cohort, Circle size corresponds to the number of fish with a valid hatching date estimate. The geographic separation of the Northern and Southern subsets is indicated by broken line.

**TABLE 1. Mean and Median Hatching Dates for Each Species, With Measures of Variability**

| Year | N[a] | Mean date | Mean Julian date | SD[b] | Median date | Min[c] | Max[d] | Range (days) |
|---|---|---|---|---|---|---|---|---|
| (a) *N. furzeri* | | | | | | | | |
| 2010 | 32 | 7 Feb | 38.2 | 18.54 | 7 Feb | 3 Jan | 6 Mar | 62 |
| 2012 | 67 | 25 Jan | 24.9 | 5.81 | 24 Jan | 14 Jan | 6 Feb | 23 |
| 2013 | 73 | 14 Feb | 45.2 | 21.01 | 10 Feb | 30 Dec | 29 Mar | 89 |
| (b) *N. kadleci* | | | | | | | | |
| 2010 | 14 | 15 Feb | 45.7 | 14.17 | 11 Feb | 27 Jan | 7 Mar | 39 |
| 2012 | 3 | | Not applicable. | | 31 Dec | 25 Dec | 2 Jan | 9 |
| 2013 | 19 | 7 Feb | 38.4 | 15.31 | 7 Feb | 17 Jan | 16 Mar | 55 |
| (c) *N. orthonotus* | | | | | | | | |
| 2010 | 22 | 25 Feb | 56.2 | 15.27 | 28 Feb | 31 Jan | 20 Mar | 45 |
| 2012 | 42 | 24 Jan | 24.0 | 7.98 | 24 Jan | 4 Jan | 14 Feb | 41 |
| 2013 | 39 | 25 Feb | 56.2 | 11.14 | 25 Feb | 27 Jan | 24 Mar | 55 |
| (d) *N. pienaari* | | | | | | | | |
| 2010 | 12 | 22 Feb | 53.2 | 17.26 | 27 Feb | 28 Jan | 20 Mar | 48 |
| 2012 | 12 | 22 Jan | 22.7 | 4.98 | 24 Jan | 11 Jan | 29 Jan | 18 |
| 2013 | 24 | 24 Feb | 55.3 | 13.50 | 1 Mar | 1 Feb | 19 Mar | 43 |

[a]Sample size.
[b]Standard deviation.
[c]Earliest detected hatching date.
[d]Latest detected hatching date.

of multiple cohort in our dataset is likely overestimated. First, we specifically sampled the full size range of fish in the field (to include the smallest and the largest fish for otolith reading). Second, otolith age reading in fish older than 30 days may potentially have lower precision (Campana, 2001). The examples of particular cases of synchronous and asynchronous hatching are illustrated in Figure 5.

We found four unambiguous cases of two separate age cohorts in a total of 58 analyzed sites (7%). In one site (site 233, Beira region; Fig. 5B), samples of each age cohort were sampled on different dates (26 February and 14 April, respectively) and datalogger readings over that period suggest that the age cohorts were separated by habitat desiccation. Each age cohort was represented by a different species (Fig. 5B). In the other three sites, all samples

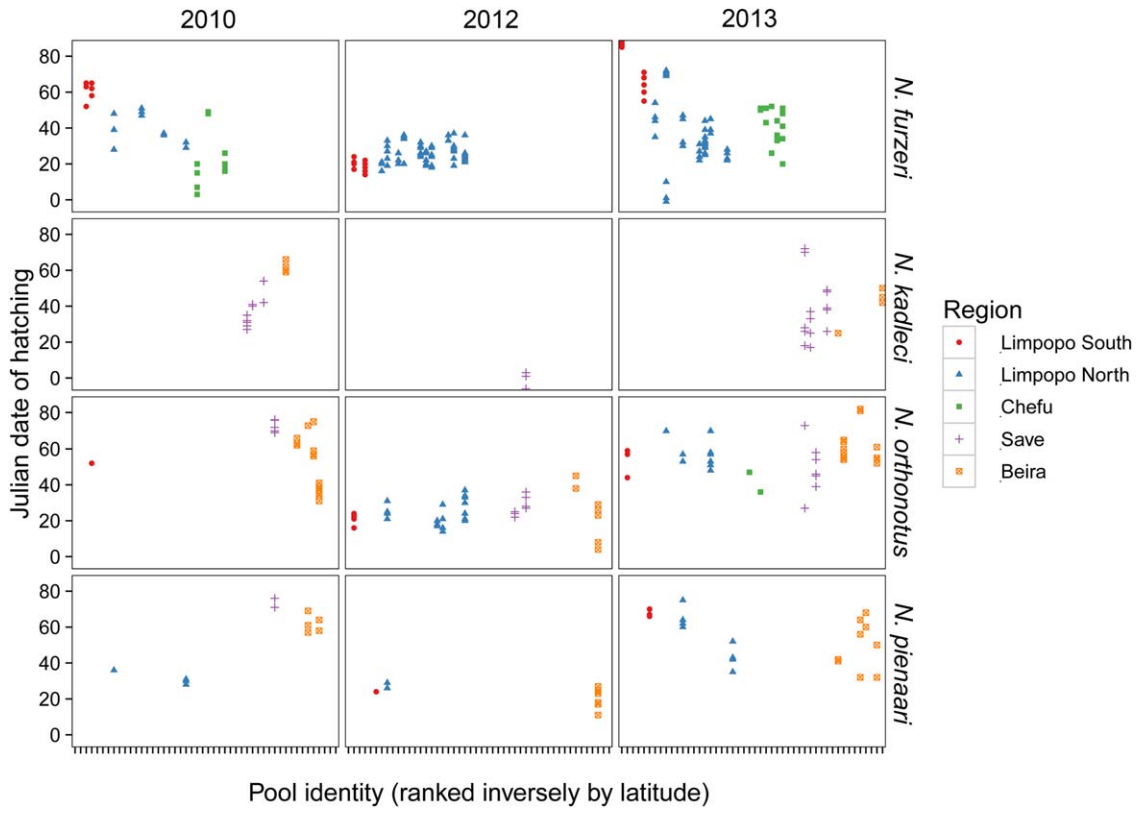


**Fig. 3.** Inter-annual variability in hatching dates across study regions and species. Populations are ranked along latitudinal gradient (south to north), with different geographical regions (consistent with different genetic clades sensu Bartáková et al., 2015) represented by different symbols.

were collected during the same date. All those sites were situated in topographically variable locations. Two cases corresponded to roadside culverts that likely accumulated water spilled over from adjacent pools (site 109 sampled in 2013, and site 108 in 2012, both in the Save region; Fig. 5D,H). One case (site 007, Limpopo north region; Fig. 5F) came from a location with three adjacent pools that may have become connected during precipitation-driven local flooding, with fish migrating between formerly separated sites.

In contrast, age estimates confirmed the existence of a single age cohort in several cases where body size distribution suggested potential multiple hatching events, either between (Fig. 5A,B) or within species (Fig. 5B,C). There were exceptional cases where hatching appeared extended, but with no distinct bi- or multi-modal pattern (Fig. 5G).

## Regional and Inter-specific Variation

There were no differences in mean hatching date among five main regions (Linear Mixed Model, LMM: $F_{4,9.6} = 0.3$, P = 0.902), with sampling year being a significant covariate ($F_{2,12.7} = 11.3$; $P = 0.002$). However, mean hatching date differed among species (LLM; species: $F_{3,141.7} = 6.0$; $P = 0.001$), consistently across study years and across all sites (year: $F_{2,18} = 16.2$; $P < 0.001$; species by year interaction: $F_{5,140.3} = 1.8$; $P = 0.123$). The mean hatching date of *N. kadleci* was 24 January (95% confidence interval [CI]: 11 Jan–6 Feb), which was significantly earlier than the mean hatching date of the other three species: *N. furzeri* (10 Feb, 95% CI: 2–19 Feb), *N. orthonotus* (15 Feb, 95% CI: 7–23 Feb), and *N. pienaari* (16 Feb, 95% CI: 16–25 Feb). This outcome

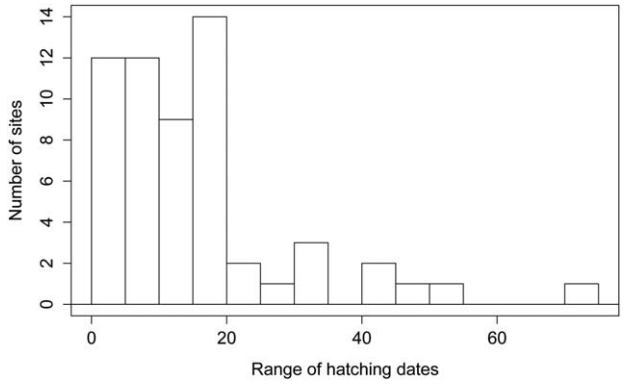


**Fig. 4.** Histogram of the absolute range (maximum – minimum) of hatching dates across all sites with hatching date estimates for at least three individual fish. The highest possible range of fish body size was selected (rather than a random subsample), maximizing inclusion of the full range of hatching dates despite relatively small number of fish analyzed per each site. Histograms based on data from sites with >3, >4, and >5 fish per site produced qualitatively identical results.

was confirmed by analyzing the southern (*N. furzeri* range) and northern (*N. kadleci* range) subsets of sites separately (Fig. 2). There was no interspecific difference in the southern subset (containing no *N. kadleci*) (LLM: $F_{2,82.8} = 1.92$; $P = 0.153$) but *N. kadleci* hatched earlier than *N. orthonotus* and *N. pienaari* in the northern subset (LLM: $F_{2,55.9} = 6.84$; $P = 0.002$). This interspecific difference may have been related to the preference of *N. kadleci* for pools that were inundated earlier or, alternatively, to within-site variation in hatching dates among sympatric species.

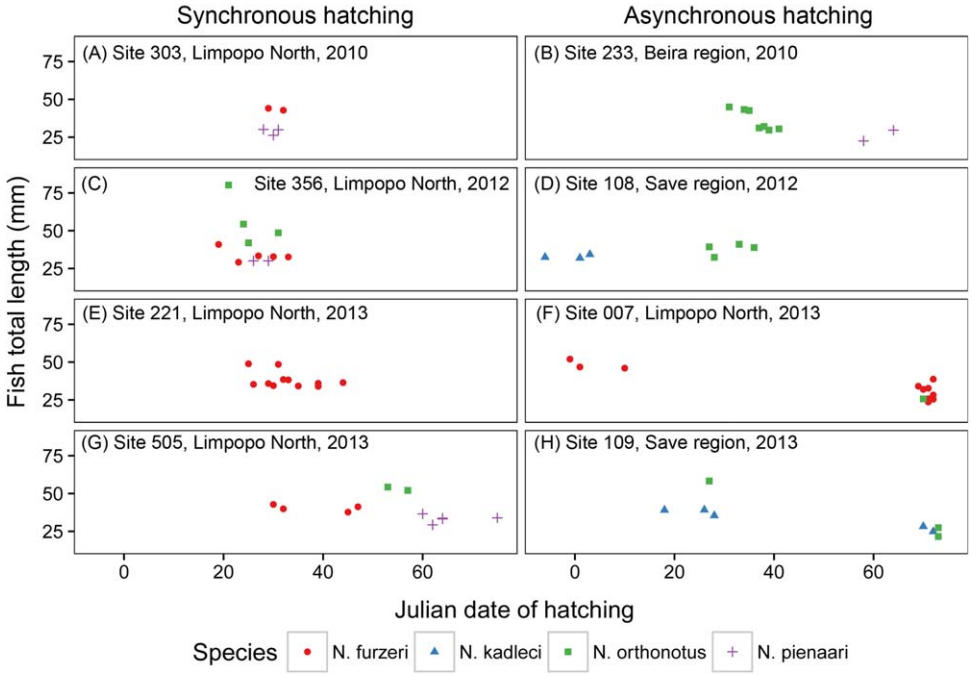


**Fig. 5.** Examples of synchronous and asynchronous hatching at particular sites visualized by bivariate plots of back-calculated hatching date and body size at time of sample collection. Different species are distinguished by different symbols.

Hence, we further examined whether their earlier hatching date could also be detected within sites. Only three sites had hatching date estimates for coexisting individuals of *N. kadleci* and another species. Site 108 (year 2012) had two age cohorts: *N. kadleci* hatched between 25 December 2011 and 4 January 2012, while *N. orthonotus* hatched between 28 January and 5 February 2012 (Fig. 5D). Site 109 (year 2013) also had two distinct age cohorts (late January and mid March hatching), but each age cohort was represented by individuals from both *N. kadleci* and *N. orthonotus* (Fig. 5H). At site 099, only three fish were analyzed and one *N. kadleci* was estimated to hatch 16 days earlier than two *N. pienaari*. Given that another six sites had estimates only for *N. kadleci*, the cause of the difference in hatching time between *N. kadleci* and other species remains ambiguous. In the southern subset (*N. furzeri* range), no interspecific variation within sites was apparent (Fig. 5A,C) but cannot be entirely rejected (Fig. 5G).

### Relationship Between Individual Age and Body Size

Individual body size was positively correlated with the estimated age of male and female fish in all species (Pearson correlations: all $P < 0.05$; n = 22–93), except for male *N. kadleci* ($P = 0.63$; n = 15) (Fig. 6). While statistically significant, the proportion of variability explained was always relatively low (11–36%; $r^2 = 0.331$–$0.603$). Among juvenile fish, body size was not correlated with estimated age (Pearson correlation: $P = 0.105$; n = 21 and $P = 0.251$; n = 7, for *N. furzeri* and *N. orthonotus*, respectively). Overall, body size cannot be used to estimate individual age.

### Regional and Local Variation in Habitat Inundation

In 2012, most pools in the southern range (Limpopo North, Limpopo South and Chefu regions) were inundated on 16 January (12 pools in total); only a single pool had different inundation regime (Fig. 7A). This large-scale synchrony corresponded with fish hatching (Figs. (3 and 6)). In most pools, fish appeared to hatch from 16 to 19 January. Age estimates from the otoliths suggested that hatching continued until the end of January, with some age estimates compatible with hatching on 6 February. A single fish was estimated to hatch on 14 January (2 days before inundation), likely due to imprecise age estimate. No regional synchrony was detected in the other regions, except in the Save region on 30 March (Fig. 7A).

In 2013, synchronous inundation in the southern range (Limpopo North, Limpopo South and Chefu regions) was evident from 11 to 14 January, with most sites flooded on 11 January. In addition, there were nonseasonal inundations during September and December 2012 (i.e., within the 2013 rainy season) in three pools that lasted between 10 and 40 days (Fig. 7B). These sites were re-inundated during main seasonal precipitation, with a desiccation period longer than 50 days between the first and second within-season inundation. The other regions showed less synchrony, although the inundations in early January were apparent across the study area (Fig. 7B). Inundation data are not available for 2010.

## Discussion

Using a set of 359 individuals, we compared the hatching dates of annual killifish embryos in the wild across and within years, regions and species, and related them to the start of habitat inundation estimated from dataloggers deposited in field sites across the entire study area. Our first prediction suggested inter-annual consistency in hatching dates, associated with the major seasonal precipitation in January. Instead, we observed the predicted hatching peak in January only in 2012. In 2010 and 2013, hatching appeared to peak later (mid February) and was temporally

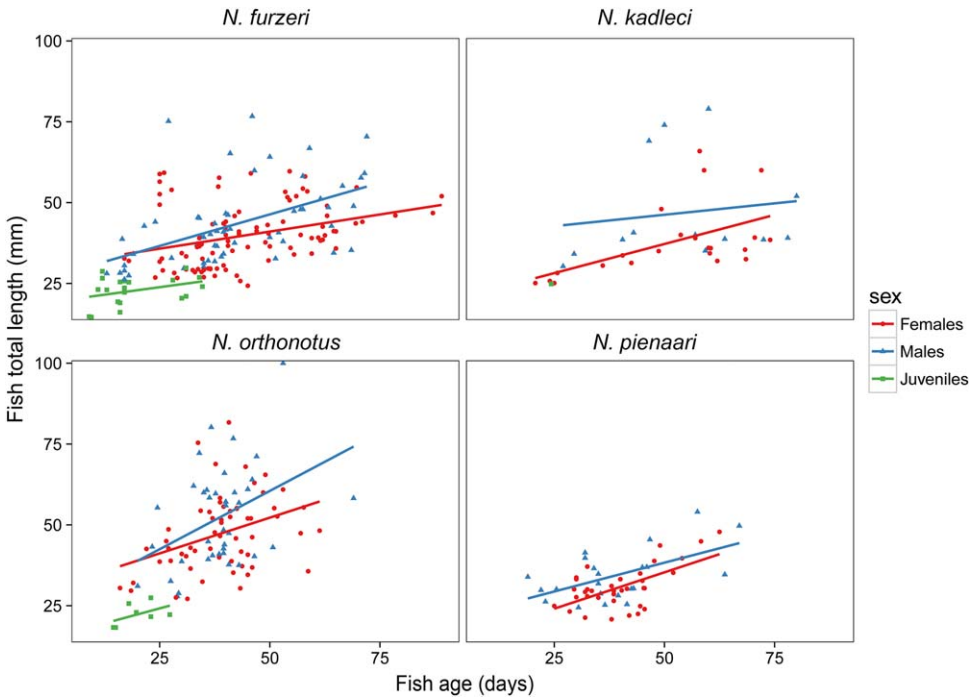


**Fig. 6.** Association between individual body size and estimated age across all samples for a given sex and species.

more protracted (Fig. 3), despite apparent synchronous inundation over the southern part of the study area on 11 January 2013 (Fig. 7B). We further predicted hatching synchrony across the entire region and within each site as well as no inter-specific differences in hatching dates. Instead, hatching synchrony was evident only in 2012, with hatching dates spread across three months in the other two study years (Fig. 3). One species (*N. kadleci*) tended to hatch earlier. The hatching at some sites was unexpectedly protracted (Figs. (4 and 5)), and multiple age cohorts were detected at three sites. Below, we discuss potential caveats and biases in our estimates of hatching dates. We confirmed that body size cannot be used as a surrogate measure of age. Finally, we confirmed our prediction of the existence of secondary pools, when the same site was desiccated and re-inundated during a single rainy season, with a new annual fish cohort/generation.

The embryo development of annual killifish in the laboratory is known to respond to a set of environmental cues, including ambient temperature and oxygen availability (Wourms, 1972c; Inglima et al., 1981; Levels and Denucé, 1988; Furness et al., 2015b; Pinceel et al., 2015; Polačik et al., 2016). While it is not known how precisely annual killifish embryo development is regulated in the wild, it is supposed that embryos modulate their development to match hatching with seasonal inundation (Watters, 2009) and, hence, minimize the risk of premature hatching after nonseasonal rains. At the same time, embryo development is affected by maternal contribution to the egg, with embryos spreading termination of their prehatching development from 3 to over 50 weeks, despite incubation in an identical developmental environment (Polačik et al., 2017).

The current understanding of embryo ecology in the wild is based on the following scenario (Wourms, 1972c; Watters, 2009; Reichard, 2015). First, embryos are deposited shallowly in the soft mud sediment of the pool. After an early phase of embryo development, embryos likely persist under anoxic conditions in a dormant stage (diapause I). Reduced oxygen content is known to cause the embryo to enter diapause I as early as 3 days after fertilization during the dispersed phase of development at the end of epiboly (Wourms, 1972c). While Inglima et al. (1981) suggested that diapause I is induced by a chemical cue from adult conspecifics to eliminate embryo development and hatching in the pool with a cohort of adult fish, we speculate that diapause I may also be an adaptation to anoxic conditions.

A second major environmental perturbation is related to the start of the aerobic phase of the embryo environment, when oxygen starts to penetrate the sediment during the later stage of desiccation (Watters, 2009). This may enable embryos to complete another phase of active development (somitogenesis, formation of neural keel, functional tubular heart) (Wourms, 1972c) and proceed to diapause II (Watters, 2009). Embryos in diapause II are extremely resistant to environmental challenges, and this is the stage where they are capable to persist up to several years (Podrabsky et al., 2010).

The exit from diapause II is likely triggered by high precipitation resulting in the saturation of the substrate with water (Reichard et al., 2015). After a short phase of development until the prehatching stage, embryos may either hatch or enter diapause III, a stage ready for immediate hatching once environmental conditions become favorable (Podrabsky and Hand, 1999; Watters, 2009). We speculate that maternal effects on embryo development influence the degree of individual sensitivity to external factors that trigger entry into or exit from any of the diapauses. Ultimately, this creates ample opportunity for maternal bet-hedging of the offspring developmental trajectories, despite the strong role of environmental conditions as for the annual killifish, the ultimate suitability of any inundation is unpredictable.

The comparison of inundation and hatching dates demonstrates that fish start to hatch almost immediately after the pool

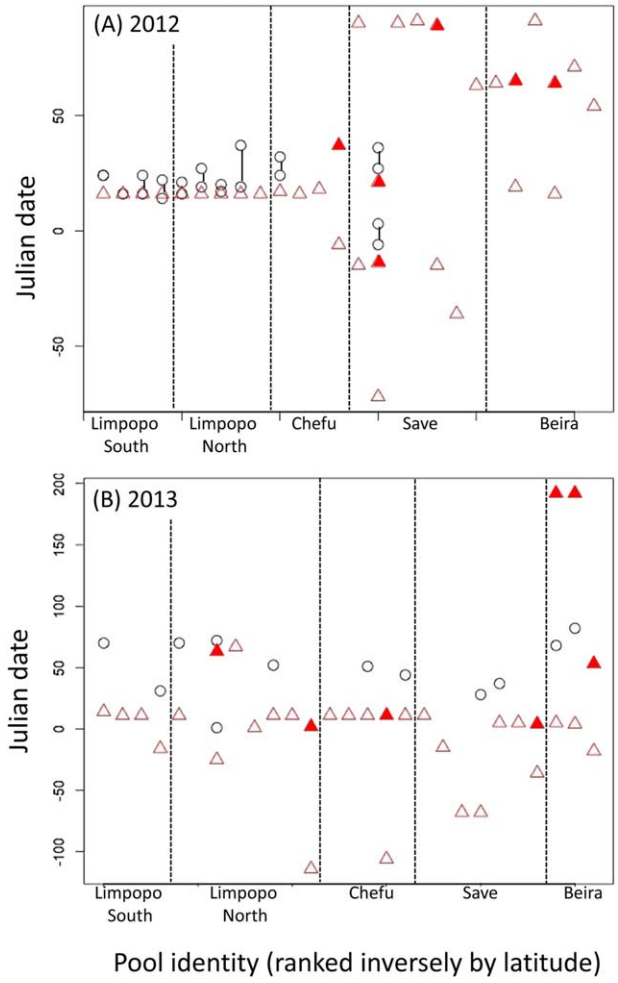


**Fig. 7.** Inundation date estimates across the study area (red triangles) and hatching date estimates (open circles) for the years 2012 (**A**) and 2013 (**B**). Sites were ranked along latitudinal gradient (south to north), with different geographical regions (consistent with different genetic clades sensu Bartáková et al., 2015) separated by dotted lines. Filled red triangles illustrate repeated inundations. Note that not all sites with inundation estimates have corresponding estimates of hatching dates.

is filled (Fig. 7). Importantly, our data also suggest that some embryos hatch several days later than the others (Figs. 3–5), and different pools vary in the propensity toward protracted hatching. We have no information on whether the embryos that hatched immediately after inundation were at a different developmental stage than embryos that hatched at a later date. While this is possible, an alternative explanation is that the sensitivity of some embryos to external cues may require inundated conditions to last for several days to trigger hatching (Furness et al. 2015b). Yet other embryos, with a different threshold may not hatch during that inundation at all, resulting in a very effective parental bet-hedging strategy (Wourms, 1972c; Pinceel et al., 2015). Such a mechanism could explain asynchronous hatching with and without desiccation, as illustrated on Figure 5.

Developmental and hatching asynchrony may also be a consequence of extrinsic (rather than intrinsic) factors. It is possible that different embryos developed in different sediment depth and environmental variability at microhabitat scale resulted in the asynchronous hatching. This would be a likely consequence of large mammals (currently mainly domestic cattle) trampling the sediment and mixing various layers (Reichard et al., 2009; Reichard, 2015), potentially pushing some eggs deeper into the substrate. Finally, some within-pool variation in hatching dates is likely associated with pool topography, where different parts of the pool are inundated at different times, and with transient connections between adjacent pools. This could explain within-site variability in hatching dates especially in years when the main seasonal rains are lighter, leading to more localized patterns of hatching. The observed patterns of inter-annual variability in hatching date synchrony, with a stronger within-site synchrony in the years with large-scale precipitation (Fig. 3), are consistent with this possibility. Establishing the exact environmental conditions that annual killifish embryos experience in the wild, either through direct embryo sampling or recording by dataloggers, would be necessary to complete our understanding of cues and triggers that affect the rate of embryo development, entry into diapause, and hatching.

Inter-specific differences in hatching dates between sympatric species were detected (Fig. 5). Older individuals of *N. kadleci* were found in the same region and, sometimes, in the same pool than those of the other species. *N. kadleci* is a member of the *N. furzeri* species group (F-clade sensu Bartáková et al., 2015) that is associated with drier conditions than the other two Mozambican *Nothobranchius* clades (represented by *N. orthonotus* and *N. pienaari*, respectively, in the present study). Most notably, *N. kadleci* is absent from the more humid coastal area, where *N. orthonotus* and *N. pienaari* inhabit numerous pools that receive relatively more precipitation (Reichard et al., 2017).

We speculate that *N. kadleci* embryos may be relatively more sensitive to hatching cues from habitat inundation (i.e., possessing finer thresholds in their reaction norms), as a consequence of stronger selection for missed opportunities to hatch. Of interest, *N. kadleci* has the shortest maturation time of all annual killifish species and, ultimately, of all vertebrates (Blažek et al., 2013), and appears to be generally adapted to extremely rapidly desiccating habitats (Reichard, 2010). A similar trend was also apparent in some populations of the closely related *N. furzeri* that coexisted with other *Nothobranchius* species (Fig. 5G). Locally co-occurring *Nothobranchius* species differ in habitat use (Reichard et al., 2009, 2017), with a tendency for *N. pienaari* to inhabit pool margins and *N. orthonotus* to be associated with deeper water. Such differences in habitat use could potentially translate into differences in the location of egg deposition sites among species. In contrast to interspecific variation in sensitivity to environmental cues, we do not consider differences in habitat use to affect interspecific variation in hatching time. Individual pools are inundated within such a short period (hours) that there is little potential for differential timing of hatching on the basis of specific location of egg deposition within a pool.

Individual body size cannot be used as an estimate of individual age. While there was a positive correlation between fish length and estimated age, the variation in individual size at any given age was up to three-fold (Fig. 6). Growth rate in *Nothobranchius* is strongly affected by social factors (dominance hierarchy) and population density (Polačik and Reichard, 2009; Polačik et al., 2011, 2016). Given that otoliths in *Nothobranchius* fish older than 90 days are difficult to read reliably for daily increments, alternative approaches need to be used to provide reliable age estimates for older fish. The reading of scale circuli is used in some fishes, although its precision for reading daily increments is

highly questionable (Szedlmayer et al. 1991); scale circuli are deposited over longer time periods and correspond to growth rate rather than age (Walker and Sutton, 2016).

One solution is to collect fish at an earlier age (to age them with sufficient precision) and return to the site at a later date to collect older fish. Older fish then can be aged by adding the elapsed time between samplings to produce an age estimate for later collection date. This procedure, however, can only be used if a single cohort, hatched over a short period, is present at the site. This condition varies across sites, species, and years, and it is necessary to obtain data from other biogeographic regions (where timing and arrival of the seasonal rains are typically more predictable) to establish whether the presence of multiple cohorts and protracted hatching is a characteristic of other *Nothobranchius* species.

In conclusion, our data suggest that hatching is a combination of environmentally determined transition from embryonic to postembryonic life and individual variation in sensitivity to environmental cues. This is manifested as a protracted hatching period of 1–20 days after habitat inundation. Ultimately, this appears to function as an effective bet-hedging strategy to spread the risk of failure of juvenile fish to complete posthatching development. If deficient precipitation does not lead to sufficiently long habitat inundation, individual variation in the propensity to hatch allows for the hatching of another set of fish during the next inundation (Podrabsky et al., 2010; Polačik et al., 2014; Furness, 2016; Pinceel et al., 2015). Functionally, maternal epigenetic contribution to the egg, perhaps provisioning the eggs with small molecules (RNA, proteins) (Podrabsky et al., 2015), rather than the genetic background of the parents (Polačik et al., 2017), appears to ensure variation in the functional response to environmental triggers. Hatching in the annual killifish is, therefore, likely a consequence of the interplay between suitable environmental conditions and individual predisposition to respond to environmental threshold cues.

## Experimental Procedures

### Fish Collection

Adult fish were collected in the wild during five expeditions (20 February – 4 March 2010, 5–16 April 2010, 23 February – 16 March 2012, 21 May – 7 June 2012, and 28 March – 16 April 2013) using dip nets and seine nets (Reichard et al., 2014), euthanized with an overdose of clove oil and stored in 96% ethanol. Individuals were selected to include the widest body size range represented in a population. The smallest and largest individuals were actively targeted to be sampled within each population. This procedure maximized the chance of including all potential age cohorts present. Total sample size per site was typically more than 30 individuals, and we could only include a small subsample for otolith reading. At the level of pools, we aimed to cover as large a geographical distribution as possible. We generally avoided sampling sites that were adjacent and selected distant and well separated sites within each region.

Fish were identified to species based on coloration and morphology. Some pools (n = 3) were inhabited by *N. rachovii*, a sister species of *N. pienaari* (Shidlovskiy et al., 2010) and its ecological vicariant (R-clade sensu Bartáková et al., 2015). We treated *N. rachovii* and *N. pienaari* as a single taxon (labeled as *N. pienaari*) in all analyses. The exclusion of *N. rachovii* from all estimates made no qualitative difference to the results and interpretation.

Sampling was performed under a license from the Mozambican Ministry of Fisheries (collection licences: DPPM/083/7.10/10, DPPM/330/7.10/10, DPPM/088/7.10/12; export licences: 133/2791/MP/2010, 191MP/2012, 238MP/2013), and general research procedures were approved by the ethical committee of the Institute of Vertebrate Biology, in accordance with the legal regulations of the Czech Republic.

### Otolith Reading

In the lab, fish were measured for total length (from the tip of the snout to the end of caudal fin) and sent to a commercial otolith reading facility (Barcelona Otolith Reading Service (BORS), Spain, EU) for age estimates. In BORS, sagittal otoliths were removed by dissection, mounted on microscope slides, and polished until daily increments were visible. Reading of each otolith was repeated at least twice; a third reading was done in cases where the first and second readings produced conflicting results. The full set of readings was provided to the authors by BORS. The precision of otolith reading was previously validated for *N. furzeri* from the age of 7–66 days (n = 14 fish), with high precision between known and estimated age (r = 0.99) and a mean error of 1.3 (SD = 1.5, maximum 6) days (Polačik et al. 2011). We acknowledge that estimates were not validated for the other three species. However, similarity in otolith structure (Polačik et al., 2011; Reichenbacher and Reichard, 2014) suggests comparable precision among species.

We used average values from multiple readings of each otolith, although one of the readings was discarded if a clear mistake was discovered during the third reading. If the inconsistency was not resolved, we discarded the specimen from the analyses, because the resulting average value would have no biological meaning. Average values were readily used for age estimates within the range of 1–4 days, given the precision of otolith readings (Polačik et al., 2011). Consequently, an age difference of a few days may derive solely from imprecise estimates while a marked difference of 1–3 weeks represents robust evidence of an extended hatching period. Overall, otolith readings gave age estimates of 8–89 days for particular individuals (mean [± SD] = 41 [±14.8] days).

Of a total sample of otoliths from 441 fish, some otoliths did not preserve at the quality required for precise reading or were impossible to read due to deformities. Some individuals were too old to provide a reliable age estimate (likely > 90 days of age). The final dataset (372 individuals, 84%) was further reduced by exclusion of the year 2008 (only a single site present) and three regions with a single site per region (all from 2012), resulting in a final dataset of 359 individual age estimates from 58 sites. The three excluded sites from remote regions fell within the range of values reported from other regions and were no outliers from the full set of samples.

### Estimates of Habitat Inundation

To obtain information on seasonal patterns of habitat desiccation and inundation, we deployed waterproof temperature loggers (HOBO UA-001-08, Onset Computer Corp., Bourne, MA, USA) at 36 sites across the entire study area. The loggers were deposited in the deepest part of the pool, 1–5 cm deep into fine bottom sediment, marked on a sketch map and supplemented by a metal

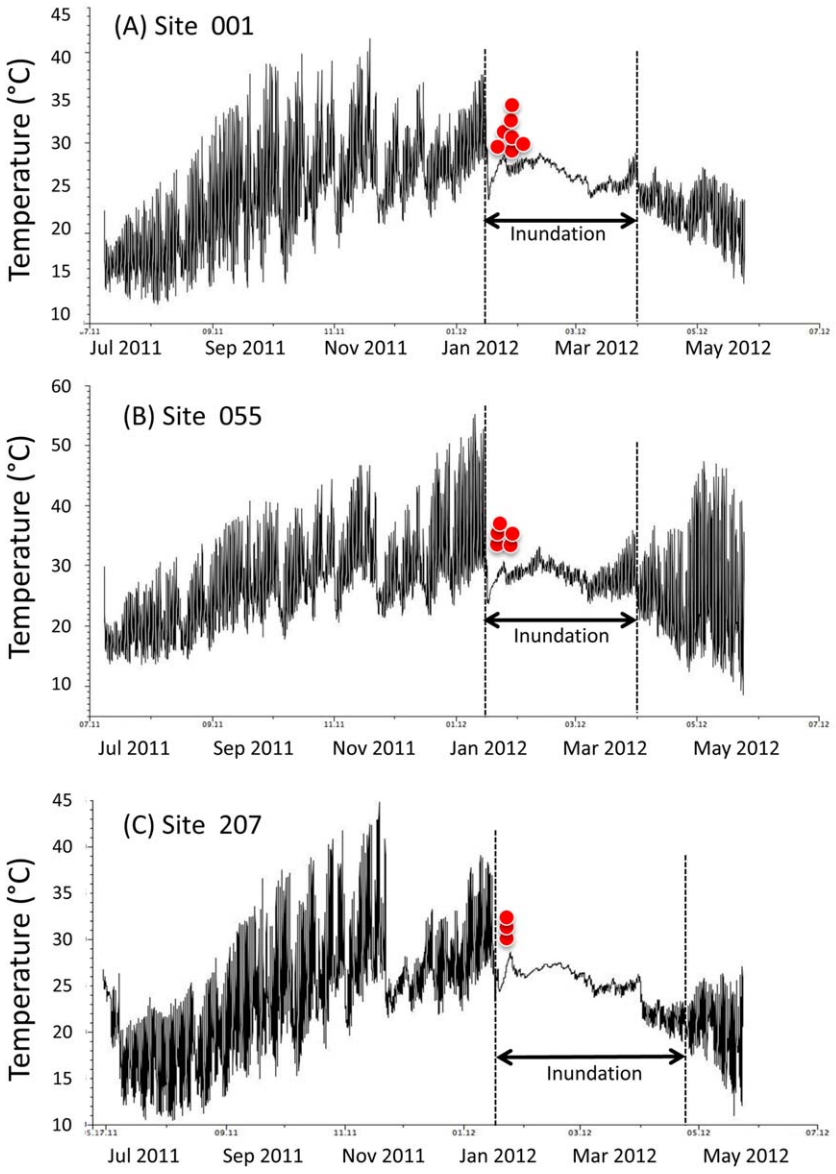


**Fig. 8. A–C:** Temperature fluctuations (logged every 3 hr) at three sites over a period of 1 year. The estimated time of habitat inundation (dotted vertical lines) and hatching dates of individual fish (red circles) are indicated.

rivet. Readings of the ambient temperature were taken every 3 hr. The loggers were retrieved using the sketch map and a metal detector (Tesoro Silver μMax; Tesoro Electronics, Prescott, AZ) during the next expedition. Some loggers were not recovered while others were destroyed and data could not be acquired. A total of 26 loggers were retrieved each year, of which 16 and 18 were successfully read in 2012 and 2013, respectively (Fig. 2). Datalogger information was not available for 2010.

Pool inundation was estimated from a sudden decline in the magnitude of daily fluctuations in ambient temperature that lasted over a protracted period and coincided with high thermal capacity of water compared with air (Terzibasi Tozzini et al., 2013; Polačik et al., 2014; Fig. 8). Likewise, pool desiccation was estimated from an increase in daily temperature fluctuations. Four estimates of every pool inundation and desiccation event were completed, one by each co-author. An average value for each site was calculated from the four estimates. When a single reading deviated from the other three estimates, only three consistent estimates were used to calculate the average inundation date. All four readings were typically consistent, with a difference of 0–2 days.

## Data Analyses

Hatching dates were obtained by subtracting estimated age in days from the date of sample collection. Dates were saved in Julian date format (score 1 was assigned to 1 January, i.e., December dates possessed negative values) and were thus normally distributed. Linear mixed models were used to compare mean hatching date across years, regions, and species. These variables were fixed factors in particular analyses. To control for dependency of data collected within each site and region, we assigned random intercepts for each site (i.e., individual pool) nested within region (i.e., five main regions: Limpopo South,

Limpopo North, Chefu, Save and Beira). Pool identity was treated as a fixed factor in the analysis of within-site variation among species. Data analysis was completed in the R statistical environment (R 3.2.4). Data were visualized using the *ggplot* library (Wickham, 2009) and mixed model analyses were completed using *lme4* library (Bates et al., 2014).

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