*Amphibia-Reptilia*

# Responses to nitrate pollution, warming and density in common frog tadpoles (*Rana temporaria*)

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**Supplementary Material**

## Appendix A. Impact of the temperature treatment on water temperature

We manipulated water temperature by placing a 100W aquarium heater on the bottom of every mesocosm in the warm treatment. Heaters were set to operate between 08:00 and 17:00 and when the water temperature was below 24ºC. There were no heaters in the ambient treatment. Data loggers were programmed to measure water temperature at two-hour intervals in 12 randomly-chosen mesocosms, six in the warm treatment and six ambient. Each mesocosm received two loggers installed at depths of 5 cm and 28 cm. Four of the mesocosms were measured during the first week of the experiment, another four during the second week, and the last four during the remaining two days until the end of the experiment.

We estimated the impact of treatment on water temperature with a mixed-effects repeated measure analysis fit by REML, including time (hours from the start of the experiment), treatment (ambient or warm), and location of the logger (shallow or deep) as fixed effects. Likelihood ratio tests were used to compare among a variety of possible random effects structures. This analysis was implemented with package nlme in R 3.1.2 (Pinheiro, J. et al. (2015): nlme: linear and nonlinear mixed effects models. http://cran.r-project.org/package=nlme).

The best model included random intercepts at the level of mesocosm, heterogeneity in the relationship with time, temporal autocorrelation with a lag of one time-step (AR1), and independent variances in the two temperature treatments. This model indicated that temperature was significantly higher in the warm treatment than in the ambient treatment (table S1; fig. S1). The effect of location was not significant because the shallow reading was warmer in mid-afternoon but slightly cooler at night (fig. S1). The linear effect of time was not significant, but there was strong temporal auto-correlation. The auto-correlation parameter, $\Phi$, was 0.902, and the variance in the warm treatment was 1.42 times greater than that in the ambient treatment (table S1).

**Table S1.** Results of a mixed-effects repeated measures analysis of the effects of time, depth of the logger, and temperature treatment on water temperature. The model is explained and random effects are described in the text of Appendix A.

| Source | Treatment level | Coefficient | SE of the coefficient | *P*-value |
|---|---|---|---|---|
| Time | | 0.0009 | 0.0043 | 0.8781 |
| Depth of logger | shallow | 1.0158 | 0.5779 | 0.0790 |
| Temperature | warm | 3.4954 | 1.0917 | 0.0014 |

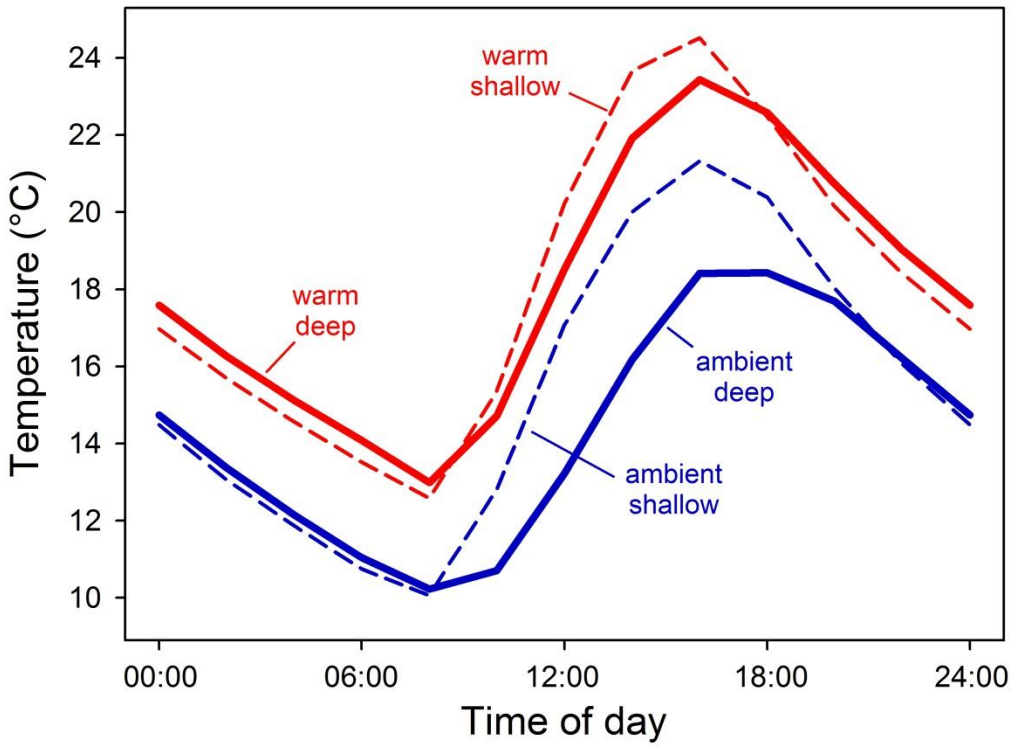


**Figure S1.** Diurnal variation in water temperature in the warm and ambient treatments, measured at two-hour intervals. Values are averaged over 12 mesocosms; four were measured for two days and the remainder for seven days.

## Appendix B. Effects of treatments on nitrate concentration

We measured nitrate concentration in eight randomly-chosen low-nitrogen and eight high-nitrogen mesocosms on 10 April, and 16 different randomly-chosen mesocosms on 19 April using a sensor array photometer (LASA®10/LASA®20 Dr Lange). We targeted nitrate because nitrification would have rapidly converted $NH_4^+$ into $NO_3^-$ under the relatively aerobic conditions of our mesocosms (Bernhardt et al., 2002, Ecosystems **5**: 419-430; Kemp and Dodds, 2002, Limnol. Oceanogr. **47**: 1380-1393). If the reading was below the detection limit, we replaced the observation with the lowest detectable value (1 mg/L $NO_3$-). The data were analyzed with a mixed-effects ANOVA, with fixed effects of date, temperature, nitrogen, tadpole density, and their interactions, and block as a random effect.

On the first sample date, the concentration of nitrate was much higher in the high-nitrogen treatment than in the low-nitrogen treatment (fig. S2). Nitrate declined sharply from the first to the second week, so that there was little difference between treatments on 19 April (fig. S2). This pattern was reflected in a highly significant date-by-nitrate interaction in the repeated measures analysis (table S2).

**Table S2.** Effect of date, temperature, nitrate, tadpole density, and their interactions on nitrate concentration in the experimental mesocosms. Date has two levels (10 April and 19 April). The random effect of block is not shown (LR = -25.6, df = 1, *P* = 1). Boldface highlights effects that were significant at α = 0.05.

| Source of variation | df | *F*-ratio | *P*-value |
|---|---|---|---|
| Temperature | 1, 16 | 0.75 | 0.399 |
| **Nitrogen** | **1, 16** | **51.56** | **0.001** |
| Density | 1, 16 | 0.13 | 0.722 |
| **Date** | **1, 16** | **40.37** | **0.001** |
| Temperature × Nitrogen | 1, 16 | 2.53 | 0.131 |
| Temperature × Density | 1, 16 | 0.12 | 0.731 |
| Nitrogen × Density | 1, 16 | 0.29 | 0.601 |
| Date × Temperature | 1, 16 | 2.64 | 0.124 |
| **Date × Nitrogen** | **1, 16** | **21.99** | **0.001** |
| Date × Density | 1, 16 | 0.17 | 0.689 |
| Date × Temperature × Nitrogen | 1, 16 | 0.81 | 0.381 |
| Date × Temperature × Density | 1, 16 | 0.18 | 0.681 |
| Date × Nitrogen × Density | 1, 16 | 0.06 | 0.817 |
| Temperature × Nitrogen × Density | 1, 16 | 0.02 | 0.906 |
| Date × Temperature × Nitrogen × Density | 1, 16 | 0.79 | 0.387 |

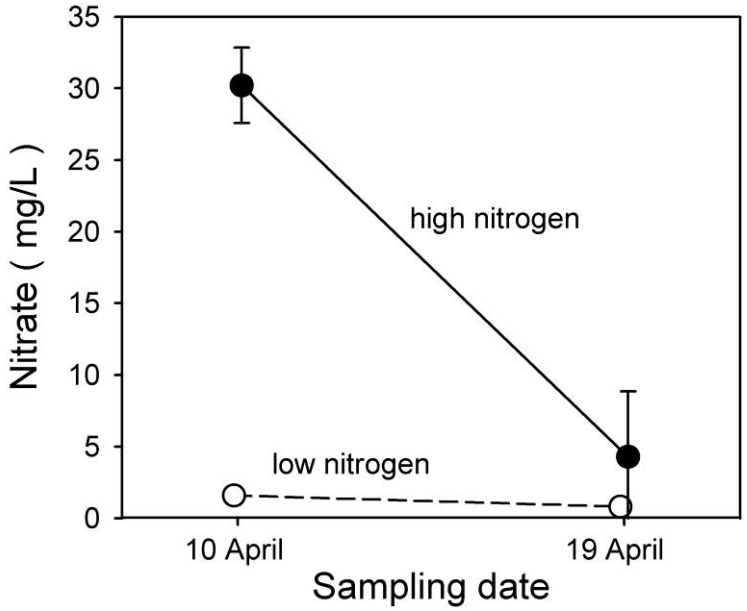


**Figure S2**. Mean (± 1 SE, *n* = 8) of nitrate concentration, pooling across non-significant density and temperature treatments.

**Appendix C. Effects of treatments on water salinity**

Salinity in all 48 mesocosms was checked on four dates (8, 11, 15, and 19 April) using a salinometer (CDC401; Hach-Lange 93 HQD series). We analyzed the data with a mixed-effects repeated measures analysis of variance, with fixed effects of temperature, nitrate, tadpole density, and their interactions, and block and mesocosm, nested within block, as random effects. The results indicated that salinity increased somewhat during the experiment, was much higher in the high-nitrogen treatment, and was higher in the warm treatment and at high density (table S3, fig. S3). Salinity in all treatments was well below levels at which impacts on tadpole performance occur (Winkler and Forte, 2011, Amphibia-Reptilia **32**:527-532).

**Table S3.** Effects of date, temperature, nitrate, tadpole density, and their interactions on water salinity in the experimental mesocosms. The random effects were both important (block: LR = 35.1, df = 1, $P < 0.0001$; mesocosm within block: LR = 10.6, df = 1, $P = 0.0012$). Boldface highlights effects that were significant at $\alpha = 0.05$.

| | df | *F*-ratio | *P*-value |
|---|---|---|---|
| Between-subjects effects | | | |
| **Temperature (Temp)** | **1, 34.6** | **93.93** | **0.0001** |
| **Nitrogen (Nitr)** | **1, 34.6** | **1793.29** | **0.0001** |
| **Density (Dens)** | **1, 34.6** | **6.08** | **0.0188** |
| Temp × Nitr | 1, 34.6 | 0.23 | 0.6346 |
| Temp × Dens | 1, 34.6 | 0.04 | 0.8335 |
| Nitr × Dens | 1, 34.6 | 0.04 | 0.8335 |
| Temp × Nitr × Dens | 1, 34.6 | 0.10 | 0.7483 |
| Within-subjects effects | | | |
| **Date** | **1, 119** | **101.00** | **0.0001** |
| **Date × Temp** | **1, 119** | **7.24** | **0.0002** |
| **Date × Nitr** | **1, 119** | **3.25** | **0.0243** |
| Date × Dens | 1, 119 | 1.91 | 0.1325 |
| Date × Temp × Nitr | 1, 119 | 0.80 | 0.4981 |
| Date × Temp × Dens | 1, 119 | 0.29 | 0.8357 |
| Date × Nitr × Dens | 1, 119 | 0.29 | 0.8357 |
| Date × Temp × Nitr × Dens | 1, 119 | 0.86 | 0.4622 |

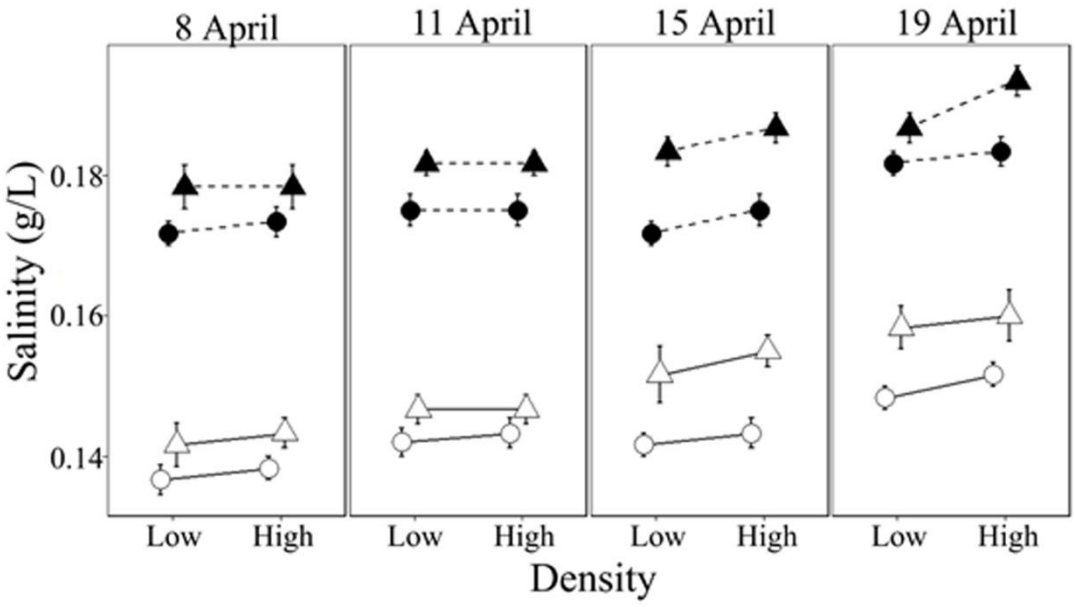


**Figure S3.** Mean (± 1 SE, *n* = 6) of salinity concentration for each experimental treatment. Circles: ambient temperature; triangles: warm. Open symbols: low-nitrate, filled symbols: high-nitrate.

## Appendix D. Effects of treatments on tadpole survival

**Table S4.** Summary of a generalized linear mixed model analysis of survival, with logit link and binomial error. The random effect of block is not shown (LR = 0.12, df = 1, *P* = 0.943).

| Source of variation | df | *F* statistic | *P-value* |
|---|---|---|---|
| Temperature | 1 | 1.99 | 0.159 |
| Nitrogen | 1 | 1.13 | 0.288 |
| Density | 1 | 1.80 | 0.179 |
| Temperature × Nitrogen | 1 | 1.77 | 0.183 |
| Temperature × Density | 1 | 2.88 | 0.090 |
| Nitrogen × Density | 1 | 2.42 | 0.120 |
| Temperature × Nitrogen × Density | 1 | 1.88 | 0.170 |

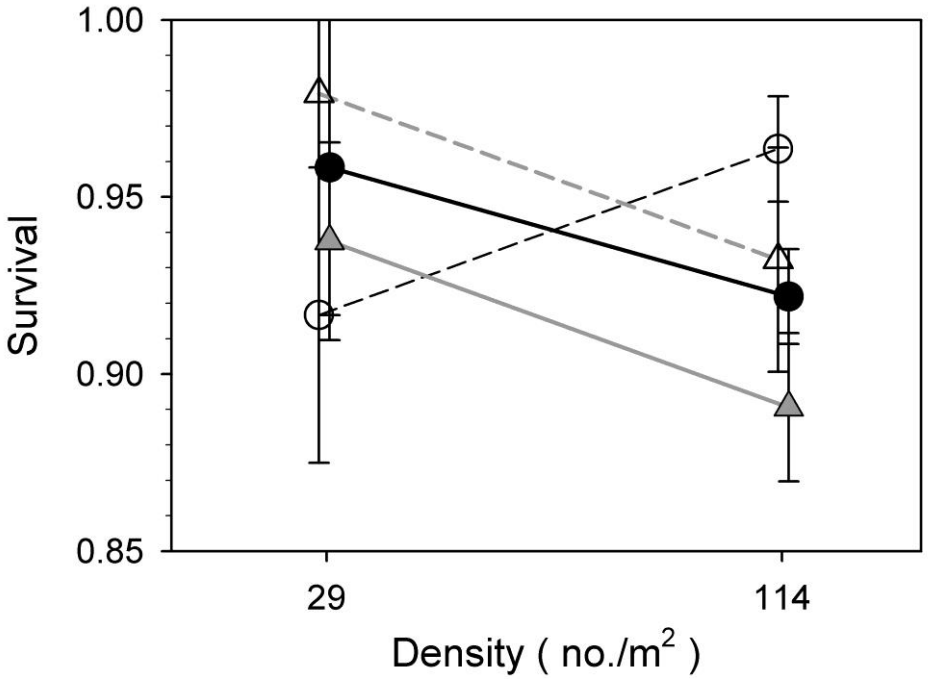


**Figure S4.** Survival of *R. temporaria* tadpoles after exposure for 16 days to variation in nitrogen concentration, tem perature, and density in outdoor mesocosms. Symbols are treatment means ± 1 SE (*N* = 6). Black lines and circles: ambient temperature; gray lines and triangles: warm. Open symbols and dashed lines: low-nitrogen, filled symbols and solid lines: high-nitrogen.