

SUBLETHAL EFFECTS OF SODIUM NITRATE ON DEVELOPMENTAL RATE  
AND BODY LENGTH IN SOUTHERN TOAD (Bufo terrestris) TADPOLES

By

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A THESIS PRESENTED TO THE GRADUATE SCHOOL  
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT  
OF THE REQUIREMENTS FOR THE DEGREE OF  
MASTER OF SCIENCE

UNIVERSITY OF FLORIDA

2003

## ACKNOWLEDGMENTS

I wish to thank my advisor, Dr. Max Nickerson, and my committee, Dr. Peter Frederick and Dr. Louis Guillette, Jr., for their invaluable guidance, advice, and support in this project. Dr. Frank Nordlie, Dr. Lauren Chapman, and Dr. Gary Butcher provided essential equipment during the course of this study.

I also wish to thank all of my friends, especially Daniel Janes, Patricia Gomez and Thea Edwards for their assistance during the course of this project and for providing adequate motivation when necessary. My gratitude is further extended to my undergraduate volunteers, without whom this project would not have been completed. I am indebted to my parents for their undying support and encouragement when morale was lacking.

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Abstract of Thesis Presented to the Graduate School  
of the University of Florida in Partial Fulfillment of the  
Requirements for the Degree of Master of Science

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August 2003

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Agricultural industries are the major contributors of nonpoint nitrate pollution in the environment. Runoff from agricultural areas ultimately resides in aquatic habitats where amphibians breed and develop in North America. Many studies have documented adverse effects of nitrates on amphibians including mortality, edema, reduced feeding and swimming activity, spinal curvature, and reduced growth and developmental rates. My study used low levels of sodium nitrate (0 mg/L, 5 mg/L, 15 mg/L, and 30 mg/L) on Southern toad tadpoles (Bufo terrestris) to determine if there was an effect on body length and developmental rate. Levels used in this experiment reflect nitrate concentrations in areas where this species develops. Tadpoles were obtained from one population and three separate clutches. I staged and measured them every other day until all tadpoles had either metamorphosed (Gosner stage 45) or died. All tadpoles were at the same Gosner stage at the beginning of the experiment. Water temperature and pH were

sampled frequently. Dissolved oxygen was measured randomly throughout the experiment.

There was no relationship among water temperature, pH, dissolved oxygen and tadpole body length. Time to metamorphosis was significantly different between 0 mg/L and 30 mg/L treatments, 5 mg/L and 30 mg/L treatments, and 15 mg/L and 30 mg/L treatments. Time to metamorphosis was not significantly different among 0 mg/L, 5 mg/L, and 15 mg/L sodium nitrate treatments. Water parameters were relatively consistent among tadpole tanks throughout the experiment. Nitrate treatment had no effect on tadpole body length (mm). It is possible that the population from which I obtained tadpoles contained animals tolerant to nitrate contamination (because of past selection favoring individuals more tolerant to nitrate). Individuals from this population may have higher phenotypic plasticity with respect to nitrate tolerance. It is also plausible that the levels used in the experiment were too low to produce effects on body length (since published studies found effects in many species at much higher levels). Nitrate treatment accelerated developmental rate. Tadpoles may have accelerated development in order to escape an unfavorable habitat. Alternatively, sodium nitrate may have stimulated developmental rate by interacting with hormones responsible for metamorphosis. Clutch had an effect on tadpole length and stage, with one clutch developing more slowly than the other two clutches and exhibiting smaller body lengths throughout most of the experiment. This result could be due to genetic factors or nongenetic factors from maternal influences such as yolk content and egg size.

CHAPTER 1  
AMPHIBIAN DECLINES AND NITRATES

**Global Amphibian Declines**

Global decline in amphibian populations is a relatively recent concern (Barinaga 1990; Phillips 1990; Dunson et al. 1992; Howe et al. 1998). Although many extinctions have occurred, amphibian declines alarm scientists because they are occurring at unnaturally high rates and because anthropogenic factors are most likely involved (Barinaga 1990; Hecnar 1995; Schuytema and Nebeker 1999a). Increased exposure to ultraviolet radiation due to ozone depletion has been studied intensively as a potential cause of decline, especially in amphibian populations that occur at higher elevations where ultraviolet radiation may have greater effects (Blaustein et al. 1994; Hays et al. 1996; Langhelle et al. 1999). Fungal and bacterial epidemics, iridoviruses, droughts, temperature changes, habitat destruction and fragmentation, exotic species, heavy metals, acid rain, pesticides and fertilizers have also been proposed as agents of amphibian decline (Barinaga 1990; Phillips 1990; Dunson et al. 1992; Howe et al. 1998; Adams 1999; Hayes et al. 2002).

Species-specific differences in response to (and tolerance of) these different agents further complicate research on amphibian declines (Rouse et al. 1999; Hayes et al. 2002). In addition, most studies focus on individual agents and their effects on amphibians rather than including multiple agents in the study. This is important since agents most likely work synergistically to adversely affect amphibian populations (Rouse et al. 1999). It is necessary to study these effects since amphibians are important predators and prey in

their ecosystems. Such studies are also necessary to effectively conserve and monitor amphibian populations (Hecnar 1995).

### **Nitrate Fertilizers**

Although nitrogen enters ecosystems from sewage effluents, industrial waste, urban runoff, atmospheric deposition, and livestock feed lots, agricultural application of nitrogen-based fertilizers remains the major nonpoint source of nitrates in the environment (Camargo & Ward 1995; Carpenter et al. 1998; Rouse et al. 1999).

Nitrogen fertilizers are widely used in the United States and Canada. In less than 50 years, the use of nitrogen fertilizer in the United States has increased from 0.45 million metric tons to more than 9.98 million tons (Lanyon 1995). Nitrogen becomes present at high levels in aquatic systems as ammonia, ammonium ion, nitrite, and nitrate. The most common form of nitrogen found in fertilizers is ammonium nitrate,  $\text{NH}_4\text{NO}_3$  (Oldham et al. 1997).

Fertilizers are applied to agricultural crops to provide them with nitrogen, phosphate, and potassium needed for plant growth. Agricultural operations tend to apply more fertilizer than necessary to the landscape, and the crops are not able to absorb all of the nutrients in the fertilizer (Hecnar 1995). This excess fertilizer is either washed (via runoff) into aquatic habitats where embryos and larvae develop; or penetrates the soil and ultimately contaminates groundwater (Hecnar 1995). Safe levels of nitrate in groundwater for humans have been designated at 10 mg/L nitrate (Camargo & Ward 1995). Exceeding this level may result in methemoglobinemia (blue-baby syndrome), cancer in humans, or neural tube defects in infants (Hecnar 1995; Rouse et al. 1999; Croen et al. 2001). Methemoglobinemia occurs when nitrate is converted to nitrite by symbiotic intestinal fauna, resulting in the oxidation of hemoglobin to methemoglobin (a

molecule that is unable to bind with oxygen). In aquatic habitats, nitrate levels below 10 mg/L produce mortality in salmonid eggs and fry (Kincheloe et al. 1979), in two species of insects (Camargo and Ward 1995), and in amphibians (Rouse et al. 1999). Therefore, when agricultural practices over-apply fertilizers, the excess ultimately ends up in aquatic habitats where amphibians breed and lay their eggs; and where embryos and tadpoles develop (Meyer-Aurich et al. 1998).

### **Effects of Nitrates on Amphibians**

Most research on the effects of nitrogen-based fertilizers has focused on lethal and sublethal effects of nitrates on embryonic and larval stages since they are considered to be more susceptible than adults to aquatic contaminants. This is due to the restriction of larvae to aquatic habitats where fertilizers can be concentrated during development; the permeability of the egg membrane; the presence of gills; and the highly cutaneous nature of their skin (Hecnar 1995). Sublethal effects of nitrate-based fertilizers include deformities, smaller body size, decreased movement, and decreased feeding activity that would make larvae more vulnerable to predators (Hecnar 1995; Watt and Oldham 1995; Xu and Oldham 1997; Laposta and Dunson 1998; Marco et al. 1999; Schuytema and Nebeker 1999a, 1999b; Rouse et al. 1999). Other indirect population effects could result if chronically exposed individuals survive to become adults and reproduce. Smaller body sizes can affect the ability of male amphibians to attract mates and smaller females have been shown to lay fewer and smaller eggs than larger females (Watt & Oldham 1995).

In general, the application of agricultural fertilizers occurs primarily in the spring when most amphibians breed and when embryos and larvae develop (Xu & Oldham 1997). Adult amphibians may, therefore, migrate over landscapes where fertilizers were

recently applied (Oldham et al. 1997). However, fertilizers' effects on adult amphibians have not been studied intensively.

The amount of fertilizer required to produce 50% mortality in test subjects varies among species and type of nitrogen-based fertilizer (ammonium, ammonium nitrate, sodium nitrate, or ammonium sulfate) (Rouse et al. 1999; Schuytema & Nebeker 1999a). Depending on the amount of fertilizer used, sublethal effects can occur such as embryonic or larval deformities, cardiac or abdominal edema, disequilibrium, small body size at metamorphosis, and decreased swimming and feeding activity (Hecnar 1995; Marco et al. 1999; Rouse et al. 1999; Schuytema & Nebeker 1999a).

The larval deformity, curling defect, refers to the curvature of the larva's tail. Curling defects occur when the vitelline membrane surrounding the embryo fails to expand during embryonic development; and forces the growing spine to curl. The underlying cause of curling is not well understood although one hypothesis suggests that pH affects osmotic potential within the vitelline membrane. Another hypothesis suggests that pH affects inhibition of a hatching enzyme that digests the vitelline membrane (Freda 1991; Laposata & Dunson 1998). Schuytema and Nebeker (1999a) found that Xenopus laevis (Anura: Pipidae) tadpoles exposed to 50.9 mg/L ammonium nitrate all exhibited curling defects. Since ammonium nitrate is commonly used in agricultural landscapes, a lower pH may be present in aquatic systems where amphibians congregate, thus potentially producing curling defects in larvae. Such deformities likely affect foraging and swimming ability in tadpoles, which could increase the likelihood of mortality due to nutritional deficiencies, predation, and higher incidence of infection resulting from poor nutritional status (Andrén et al. 1988). Population studies relating to this sublethal effect

of low pH have not yet been conducted, but are necessary to determine long-term survivability of species. Other deformities of low pH such as tail retention and limb malformations in metamorphs would most likely increase predation risk because they would decrease mobility (Xu & Oldham 1997).

Slow growth rates have been found in many amphibian species exposed to varying levels of nitrate (Hecnar 1995; Xu & Oldham 1997; Schuytema & Nebeker 1999a). Hecnar (1995) proposed that a small size at metamorphosis was related to decreased foraging as a direct result of methemoglobinemia in amphibian larvae. Methemoglobinemia would cause decreased oxygen transport in larvae and would reduce activity levels, including foraging. Small size at metamorphosis may be avoided if the larva undergoes a longer metamorphic period (Larson et al. 1998). However, both small size at metamorphosis and a long metamorphic period carry with them the costs of increased susceptibility to predation and desiccation (Alford and Harris 1988). Smaller individuals may not reproduce as early as larger individuals, thereby reducing fecundity (Alford and Harris 1988) and may have higher predation risks, higher rates of water loss, and a smaller range of viable prey items, all serving to decrease chances of survival (Crump 1980). A long metamorphic period may expose larvae to desiccation if larvae develop in ephemeral habitats and do not metamorphose before the habitat dries up (Hecnar 1995).

### **Summary**

Researchers are investigating many possible causes of amphibian declines worldwide, particularly anthropogenic factors. Nitrate fertilizers are heavily used in agricultural practices. Water runoff of these fertilizers into amphibian habitats may be negatively affecting populations. Laboratory studies on several amphibian species using

different nitrate fertilizers have resulted in tadpoles with slower growth, smaller size at metamorphosis, reduced feeding and swimming activity, edema, curling defects and mortality. Studies need to focus on how nitrate contamination may be affecting amphibians in the wild. Mesocosm studies are needed that mimic temporary pond communities. Such studies may elucidate direct and indirect effects of nitrates on amphibians. In addition, since there is ample evidence that nitrates produce adverse effects on amphibians, research should concentrate on determining the underlying mechanisms producing these effects. It is also essential to compare amphibian populations and species existing in high-usage areas of nitrate fertilizer with those existing in areas with little or no nitrate fertilizer usage. Such studies may reveal possible adaptive tolerance of species exposed to constant levels of nitrate.

CHAPTER 2  
SOUTHERN TOAD (Bufo terrestris) AND TADPOLE DEVELOPMENT AND  
GROWTH

**Natural History**

The southern toad Bufo terrestris is one of six species belonging to the americanus toad complex, the most broadly distributed group of the Bufo genus (Anura: Bufonidae) in North America. It is a common species that exists in the Coastal Plain of the United States from Louisiana south to Florida and north to the extreme southeast of Virginia. An isolated population is also found in South Carolina (Blair 1972, Conant and Collins 1998). Bufo terrestris is found throughout mainland Florida and the lower Florida Keys. Toads inhabiting the Keys have a noticeably smaller body size than those on the mainland. The typical snout vent length of mainland toads is 80 mm. In Florida, the southern toad is found in mesophytic hammocks, scrubs, wet prairies, pinelands and urban and agricultural areas (Duellman and Schwartz 1958). It is nocturnal, spending the daytime hiding in burrows and the nighttime foraging (Conant and Collins 1998). Its diet consists mainly of invertebrates, particularly beetles (Duellman and Schwartz 1958). In Florida, B. terrestris breeds from March through August in any lentic body of water. Females are capable of laying thousands of eggs contained in two gelatinous strings. The strings are typically attached to submerged vegetation and multiple females may lay their eggs in the same area (Martof et al. 1980). Eggs hatch after a few days; and tadpoles school together in groups of between 100 and 1000 individuals (McDiarmid and Altig 1999). Bufo terrestris tadpoles prefer warmer temperatures ( $> 30^{\circ}\text{C}$ ) in their aquatic

habitats (Noland and Ultsch 1981). Tadpoles metamorphose into 7 to 21 mm toadlets in approximately 1 to 2 months (Martof et al. 1980).

### **Tadpole Metamorphosis: Growth and Development**

Metamorphosis is the change of an aquatic tadpole into a terrestrial adult. During this process, radical external and internal morphological and physiological changes occur primarily due to changes in several hormone levels including thyroid hormone ( $T_3$  and  $T_4$ ), thyroid-stimulating hormone (TSH), prolactin (PRL), corticosterone (B), aldosterone, deoxycorticosterone, estradiol ( $E_2$ ), and testosterone (T). Many other hormones (e.g. melatonin, somatostatin, and gonadotropin-releasing hormone) may also influence amphibian metamorphosis but conclusive scientific evidence is lacking (Shi 2000). Factors such as temperature (Hayes et al. 1993), water amount, light, and density (Shi 2000) may also influence hormone levels.

Tadpoles typically exhibit a period of maximum growth after hatching from the egg (Gosner Stages 25-41) that is accompanied by little development. During metamorphosis (Gosner Stages 42-46), however, significant development occurs while little growth is obtained (McDiarmid and Altig 1999). Many factors affect developmental and growth rates including photoperiod, temperature, food abundance and quality, tadpole density, pH, and oxygen concentration. Temperature produces the greatest effect on development and growth rates. Lower temperatures typically produce slower growth and developmental rates in anuran larvae with developmental rate being affected more than growth rate (Harkey and Semlitsch 1988; Herreid and Kinney 1967). Tadpoles raised at lower temperatures, however, are usually larger than those raised at higher temperatures since developmental rate is more sensitive to temperature than growth rate (Harkey and Semlitsch 1988; McDiarmid and Altig 1999; Smith-Gill and Berven 1979). Bufo

terrestris tadpoles prefer the warmest temperatures in their aquatic environment; and often exhibit body temperatures greater than 30°C. This confers faster developmental times to reach metamorphosis before pond desiccation since this species typically breeds in ephemeral waters (Crump 1989; Noland and Ultsch 1981). Temperature also affects thyroid hormone, a major hormone producing metamorphosis in anurans. Lower temperatures slow down metamorphosis induced by thyroid hormone by requiring much higher levels of thyroid hormone in order for metamorphosis to occur (Harkey and Semlitsch 1988; Shi 2000). Therefore, variations in temperature can have significant effects on growth and developmental rates in tadpoles (Smith-Gill and Berven 1979).

Tadpole density affects growth and developmental rates in that individuals raised in low-density conditions with high food availability tend to maximize growth rates and decrease developmental rate to achieve a maximum body size at metamorphosis. However, individuals raised in high-density conditions where food abundance is low tend to exhibit lower growth rates and higher developmental rates. These individuals generally achieve a smaller body size at metamorphosis. Higher densities tend to affect growth rate more than developmental rate (Pough and Kamel 1984; Smith-Gill and Berven 1979; Wilbur and Collins 1973). In high food availability conditions more resources are allocated to growth. In low food conditions more resources are allocated to development to escape unfavorable conditions (Alford and Harris 1988). Individuals from high food conditions will metamorphose later at larger body sizes while the majority of individuals from low food conditions will metamorphose earlier at a smaller body size (McDiarmid and Altig 1999).

As with temperature and density, pH can affect developmental and growth rates, thus affecting size at metamorphosis. Low pH levels produce slower growth (Beebee 1986; Cummins 1986; Freda and Dunson 1986; Grant and Licht 1993; Warner et al. 1991) and developmental rates (Cummins 1986; Grant and Licht 1993) in tadpoles of several anuran species. Metamorphs exposed to low pH have exhibited tail retention and malformed limbs (Xu and Oldham 1997). Additionally, Beebee (1986) determined that small tadpoles were more susceptible to adverse effects of low pH than large tadpoles. Low pH also affects fertilization in adult Rana pipiens by decreasing sperm motility (Schlichter 1981).

CHAPTER 3  
SUBLETHAL EFFECTS OF SODIUM NITRATE ON DEVELOPMENTAL RATE  
AND BODY LENGTH IN SOUTHERN TOAD (Bufo terrestris) TADPOLES

**Introduction**

Sublethal effects of various contaminants such as deformities, smaller body sizes at metamorphosis, and slower growth and developmental rates have been documented in many larval amphibians (Hecnar 1995; Xu & Oldham 1997). Sublethal effects have resulted specifically from sodium nitrate, a common type of agricultural fertilizer that appears in aquatic habitats due to runoff. Schuytema and Nebeker (1999a) found that 578 mg/L nitrate-nitrogen from sodium nitrate produced 50% mortality in Pseudacris regilla embryos (Anura: Hylidae). Mortality was attributed to the nitrate ion rather than the sodium ion since P. regilla embryos required much less ammonium nitrate (25 mg/L NH<sub>4</sub>-N) to produce 50% mortality. Additionally, 9% of the surviving embryos from the sodium nitrate treatment regime exhibited edema and curling defects. Smaller lengths in P. regilla and Xenopus laevis (Anura: Pipidae) were seen at 111 mg/L nitrate-nitrogen from sodium nitrate (Schuytema and Nebeker 1999a). Mortality occurred in all Rana aurora (Anura: Ranidae) embryos at 918 mg/L nitrate from sodium nitrate. Embryo body length in this species was adversely affected at <29.1 mg/L nitrate-nitrogen (Schuytema and Nebeker 1999b).

Sodium nitrate negatively affected growth rate and survival of Bufo bufo tadpoles at concentrations of 40 mg/L and 100 mg/L of the nitrate ion. Tadpole size increased with lower concentrations of the nitrate ion (Baker and Waights 1993). A concentration

of 16.45 mg /L nitrate-nitrogen (from potassium nitrate) produced 50% mortality in Rana pretiosa. Other effects included reduced feeding, lethargy, disequilibrium, edema and curling defects at 16.45 mg/L or higher. The 10 mg/L nitrate maximum in U.S. drinking water was deemed toxic to Rana pretiosa (Marco et al. 1999). Hecnar (1995) found that 13.6 and 39.3 mg/L nitrate (from ammonium nitrate) was sufficient to produce 50% mortality in two different populations of Bufo americanus tadpoles. At 2.5 mg/L nitrate, all tadpoles of three anuran species displayed less feeding and swimming activity.

Anuran species are affected differently by contaminant concentrations (Hecnar 1995; Marco et al. 1999). In this study, I exposed Bufo terrestris tadpoles to low levels (0, 5, 15, and 30 mg/L ) of sodium nitrate to determine if these levels produced adverse effects on body length and developmental rate. These concentrations are 0, 0.824, 2.472, and 4.944 mg/L nitrate-nitrogen, respectively, based on molecular weight. These levels were chosen because they represent nitrate levels found in Bufo terrestris breeding habitats in Florida. I chose sodium nitrate as a contaminant since it is used frequently in agricultural practices. From available water data, pond nitrate-nitrogen amounts in Florida typically range from 0.1 to 1 mg/L although values of 3 mg/L or higher have been reported (United States Geological Survey. NWISWeb Data for the Nation. <http://waterdata.usgs.gov/nwis>. Accessed April 2004). Furthermore, since very few studies have used such low nitrate-nitrogen levels, this study will help determine a nitrate toxicity range for this species.

I predicted that adverse effects would be more dramatic in the higher sodium nitrate treatment levels, and that adverse effects would be manifested in tadpoles as shorter body lengths and retarded development. Most of the tadpoles in previous experiments have

developmental times longer than 60 days. It is known that species are influenced differently by varying types and concentrations of sodium nitrate. Perhaps larval period length also influences how greatly a tadpole is affected by a contaminant since individuals with faster growth display higher metabolic rates.

## **Materials and Methods**

### **Animals**

I obtained seven pairs of amplexing Bufo terrestris adults at approximately 0200 hours on the morning of 1 June 2002. Pairs were collected from a retention pond in a commercial area in Gainesville, Florida. Amplexing pairs were placed in separate plastic shoe boxes with approximately 2.54-3.81 cm of standing water from their native pond. Each of the seven pairs of Bufo terrestris laid eggs in the hours among time of capture and 1100 hours on 1 June 2002. Adults were removed from the plastic containers and released at their site of capture. One clutch of eggs perished due to a possible fungal infection. At 2030 hours on the same day, the remaining six clutches of eggs were separately placed into 55-liter containers. Before egg introduction, the containers were filled with approximately 47 quarts of chlorine-free tap water. Each container was also furnished with two air pumps to provide adequate oxygen flow in the water. Embryos remained in these containers until they reached a feeding and free-swimming tadpole stage, Stage 25 of the Gosner (1960) staging system, at which time I selected tadpoles for the experiment.

Using a lottery, I randomly selected three of the six clutches to be used in the experiment and released the remaining three clutches of tadpoles to their native pond. I used a total of 180 tadpoles in the experiment (60 tadpoles from each clutch). Tadpoles were selected for the experiment by dipping small fish nets in different areas in the clutch

containers. I placed five tadpoles from each clutch into each of the 12 tanks, providing a total of 15 tadpoles per tank.

### **Materials**

I used 0 mg/L, 5 mg/L, 15 mg/L and 30 mg/L sodium nitrate ( $\text{NaNO}_3$ ) as my treatment levels. These levels as nitrate-nitrogen are 0 mg/L, 0.824 mg/L, 2.472 mg/L, and 4.944 mg/L, respectively, calculated by molecular weight of nitrogen present in  $\text{NaNO}_3$ . Levels chosen for use in this experiment represent the low range of nitrate concentrations in Florida waters. Stock solutions of sodium nitrate were created immediately before the experiment so that each time water was to be changed, each tank would receive only 2 mL of stock solution from its respective treatment level. Another scientist labeled the solution bottles either E, F, H, or J so that I was blind to the concentrations.

On 3 June 2002, I obtained twelve 10-gallon glass aquaria to house *Bufo terrestris* tadpoles. Each treatment had three replicates, producing a total of twelve aquaria. I washed and scrubbed the tanks with a diluted bleach solution (~10% bleach) and then thoroughly rinsed them with fresh tap water. The next day, the tanks were again rinsed with fresh tap water and allowed to dry. On 5 June 2002, I set up the twelve 10-gallon tanks in an outdoor, open-air, covered setting. This setting provided tadpoles with ambient temperatures and some direct sunlight throughout the experiment but prevented the addition of rainwater to the tanks. I set up three 1-gallon, cloth painting sieves in each tank. Each sieve separated tadpole clutches within the tank. Sieves were attached to the top of the tanks using office clips and the tanks were covered with aluminum mesh to minimize the introduction of detritus and to prevent possible predation by insects. A 151-liter glass tank that was filled with tap water and constantly had air running through

it using two air pumps served as a water reservoir that would be used to replenish tank water. Tadpoles were fed rabbit pellets *ad libitum* throughout the experiment.

### **Experimental Design**

Using a lottery system, tanks were randomly assigned to one of four treatments: H, E, F, or J, representing 0 mg of sodium nitrate per liter, 5 mg/L, 15 mg/L, or 30 mg/L, respectively. Clutch order within each tank was also randomized using a lottery so that clutch order and clutch neighbors within each tank would vary among tanks. I labeled each tank with its designated treatment letter and clutch order (e.g., 1 2 3 or 1 3 2). I assigned tanks a position in the lab setting and randomly moved them every other day throughout the experiment using a lottery system. Clutch order within each tank did not change during the course of the experiment. Seven liters of water were added to each tank followed by two mL of sodium nitrate stock solution (either E, F, J, or H, depending on each tank's previous random assignment). Tanks were agitated to thoroughly mix the solutions in the water.

On 6 June 2002 (5 days after egg collection), all tadpoles were measured and staged. Body length (mm) was measured from the tip of the snout to the posterior part of the body where the tail's lateral line joined the body. Developmental staging of tadpoles was performed using a dissecting microscope and the Gosner (1960) staging system. I performed body length measurements and staging every other day. In order to stage and measure tadpoles, I removed each tadpole from its treatment tank and placed it on a water-saturated cloth. Body lengths were determined using calipers and staging required that the tadpoles be placed under a bright light for microscope use. Tadpoles were typically out of water for one minute to obtain body length and stage data.

Tank water was changed every other day, on the days when tadpoles were not staged or measured. Before changing water for each tank, tadpoles in each sieve were transferred to a small, plastic weighing boat by gently reversing the sieve. The tanks were rinsed with high-pressure water and sieves were also rinsed out to remove leftover food. Again, seven liters of water were added per tank, followed by two mL of the appropriate nitrate solution. Water that was added to the tanks was obtained from tap water that had been sitting in the 151-liter tank for approximately 48 hours with air pumps aiding in chlorine removal. Tadpoles were added to each sieve and each sieve was placed in its corresponding position in its tank according to the previously arranged clutch order. One pellet of rabbit food was added to each clutch sieve and the tanks were again covered with aluminum mesh.

Water, pH, and temperature per tank were measured frequently throughout the experiment. On days when the water was changed, pH and temperature were measured twice: once before changing the water and once after adding new water and nitrate concentrations. This provided a range for these parameters during the experiment. Furthermore, the use of maximum/minimum temperature thermometers provided daily temperature ranges for one tank in each treatment. Every day the maximum/minimum thermometers were placed in a new tank within the same treatment level. Dissolved oxygen was measured randomly during the experiment.

I checked tanks daily for tadpole mortality and abnormalities such as edema, erratic swimming, and body malformations. I recorded deceased or deformed tadpoles, noting treatment level (E, F, H, or J) and clutch number. Deceased tadpoles were removed from the experiment and a final body length and stage, if possible, were recorded. Any

tadpoles showing abnormalities were noted yet remained in the experiment. Towards the end of the experiment, each tank was also checked daily for new metamorphs (Gosner Stage 42, forelimb emergence). The presence of metamorphs was recorded, but they were only removed from the experiment when they reached Gosner Stage 45, when Bufo terrestris tadpoles have fully functioning lungs (Wassersug and Seibert 1975). The experiment lasted a total of 33 days, until all of the tadpoles had either reached Gosner Stage 45 (four limbs present, tail stub present, terrestrial stage) or died.

### **Statistical Analysis**

I calculated average body lengths per clutch in each tank. A square root transformation was performed to linearize the data. Comparisons of body length among clutch and treatment were performed over time using a mixed linear model in SAS. This determined whether clutch, treatment, or tank influenced body length in tadpoles. Water, pH, and temperature were also compared among tanks in the mixed linear model to determine any possible relationship with tadpole body length. Time to metamorphosis (days) was analyzed per tadpole using a mixed linear model as well. Stage 42 (forelimb emergence) was considered metamorphosis since this stage is easily recognizable.

### **Results**

One clutch of eggs perished, presumably due to a fungal infection, as noted by a white, opaque covering on the eggs. There was no significant difference in body length ( $F = 0.79$ ,  $p = 0.5063$ ) among treatments (Fig. 3-1) or among tanks ( $p = 0.28$ ). There was a clutch effect on body length ( $p = 0.0006$ ) over time (Figs. 3-2 - 3-6). Figure 3-5 excludes an outlier tadpole in the 15 mg/L treatment. This tadpole remained at Stage 25 throughout the entire experiment. Elimination of this tadpole produced much lower standard errors. Towards the end of the experiment this individual's body began swelling

and became transparent. On Day 28, body length (BL) of this tadpole was 10.29 mm while its body width (BW) was 8.56 mm. On Day 31, its BL was the longest at 11.91 mm with the widest BW of 12.00 mm. On Day 33, it had a BL of 10.86 mm and a BW of 10.30 mm. Ultimately, its body swelling produced a width practically the same dimension as its length, an abnormal feature for a typical tadpole in this species. Body lengths of Clutch 2 were typically smaller than Clutches 1 and 3. However, after the elapse of three-quarters of the experimental time, Clutch 2 in each treatment typically attained an equal or greater size than either or both Clutches 1 and 3. This typically occurred when Clutches 1 and 3 were in metamorphic climax.

Time to metamorphosis was significantly different among control and 30 mg/L tadpoles ( $p = 0.0346$ ), 5 mg/L and 30 mg/L tadpoles ( $p = 0.0334$ ), and 15 mg/L and 30 mg/L tadpoles ( $p = 0.0499$ ). Time to metamorphosis was not significantly different among control and 5 mg/L tadpoles ( $p = 0.9787$ ), control and 15 mg/L tadpoles ( $p = 0.6814$ ), and 5 mg/L and 15 mg/L tadpoles ( $p = 0.6954$ ). Average time to metamorphosis per treatment is depicted in figure 3-7. Figure 3-8 depicts the average Gosner stage per treatment per day. A clutch effect on time to metamorphosis was not present ( $p = 0.2190$ ). Figures 3-9 - 3-13 depict mean Gosner Stage of tadpoles by clutch over time. Figure 3-12 excludes the outlier tadpole from Clutch 1 which maintained Gosner Stage 25 throughout the entire experiment.

Dissolved oxygen appeared constant among tanks as well (Table 3-1).

Temperature and pH did not vary significantly during the course of the experiment and exhibited no apparent relationship with tadpole body length ( $p = 0.2810$ ,  $p = 0.3628$ ).

These environmental factors are known to influence life history traits such as size and developmental rate.

Days 13 and 26 had the highest temperatures at about 31°C and 30°C (Figs. 3-14 - 3-16). Differences in maximum and minimum treatment temperatures can be attributed to variation in thermometers and tank placement. Some tanks may have received more sunlight on a given day than other tanks. Possible confounding effects of the latter variability were minimized in the experiment by randomly moving tank position every other day.

Mortality was unequal among treatments. Mortality was 42.2%, 17.8%, 15.6%, and 28.9% in the sodium nitrate treatments from lowest to highest concentrations. Clutch 1 had the highest mortality and Clutch 3 had the lowest mortality overall (Fig. 3-17). Mortality was also present in each replicate (tank) with no apparent pattern (Fig. 3-18). No replicate exhibited mortality of all 15 tadpoles. Clutch 1 tadpoles had higher mortality than Clutch 2 or 3 tadpoles, regardless of treatment regime, and Clutch 2 tadpoles had higher mortality than Clutch 3 tadpoles in all treatments with the exception of the 5 mg/L sodium nitrate treatment (Fig. 3-19). In this instance, Clutch 2 and 3 tadpoles had equal mortality (one tadpole each). Higher proportions of mortality in the control and 30 mg/L treatments are reflected in lower proportions of total metamorphs throughout the experiment (Figs. 3-20 – 3-23). Proportion of metamorphs was higher for 5 mg/L and 15 mg/L treatment levels due to decreased amount of mortality. Metamorphs began appearing by Day 16 of the experiment. Clutch 1 tadpoles typically metamorphosed before Clutches 2 and 3 (Fig. 3-24). The majority of Clutch 1 tadpoles metamorphosed among Days 16 and 18 with the greatest proportion occurring on Day 16.

The majority of Clutch 2 tadpoles metamorphosed among Days 18 and 20 with the greatest proportion occurring on Day 20. The majority of Clutch 3 tadpoles metamorphosed among Days 18 and 20 also. Unlike Clutch 2, however, the greatest proportion of metamorphs for Clutch 3 occurred on Day 18.

### **Discussion**

Concentrations of 5 mg/L, 15 mg/L and 30 mg/L sodium nitrate (0.824 mg/L, 2.472 mg/L, and 4.944 mg/L nitrate-nitrogen) produced no observable effects on body length in Bufo terrestris tadpoles. Tadpoles in different tanks had statistically similar body lengths, suggesting that variation as a result of tank setup was minimal, if present at all. Tadpoles of different treatment regimes exhibited statistically similar body lengths throughout the course of the experiment. Higher concentrations of nitrate can produce negative effects on body length in this species as seen in other literature (Hecnar 1995; Schuytema and Nebeker 1999a and 1999b). However, exposing earlier stages (egg and embryo) of Bufo terrestris to the experimental concentrations may produce the proposed lethal and sublethal effects since earlier stages may differ in their susceptibility to nitrate contamination (Xu and Oldham 1997). Temperature and pH, factors known to greatly influence body length and developmental rate, did not vary greatly among tanks during the course of the experiment.

Time to metamorphosis differed among the 30 mg/L sodium nitrate treatment and all other treatments. Tadpoles exposed to 30 mg/L sodium nitrate obtained Gosner Stage 42 in the shortest amount of time. Tadpoles exposed to 15 mg/L sodium nitrate achieved forelimb emergence before 5 mg/L and control tadpoles. Control tadpoles reached Gosner Stage 42 before 5 mg/L tadpoles. These data suggest that higher concentrations of sodium nitrate (30 mg/L and 15 mg/L) accelerated developmental rate in Bufo

terrestris tadpoles. It is possible that the tadpoles in the 30 mg/L treatment increased developmental rate to escape the unfavorable habitat created by sodium nitrate. Another possibility is that a hormetic response occurred such that these low levels of sodium nitrate stimulated developmental rate by interacting with hormones responsible for metamorphosis. Hormesis refers to stimulatory effects of contaminants at low doses followed by inhibitory effects at higher contaminant levels (Bailer et al. 2003).

Contaminants such as cadmium, polycyclic aromatic hydrocarbons, and dioxin have been shown to produce hormetic effects in some species (Calabrese and Baldwin 2003).

Boone et al. (2001) found that the insecticide, carbaryl, accelerated development in Rana clamitans tadpoles. Although a faster developmental rate may be beneficial in instances where tadpoles are in a habitat that will quickly dry up, it may produce smaller body sizes at metamorphosis that could have negative effects on fecundity and survival (Alford and Harris 1988; Wilbur and Collins 1973).

All tadpoles used in the experiment originated from a single population in the wild. Hecnar (1995) reported that 13.6 mg/L of  $\text{NH}_4\text{-N}$  was sufficient to produce 50% mortality in one population of Bufo americanus tadpoles while another population required 39.3 mg/L of  $\text{NH}_4\text{-N}$  to produce 50% mortality. The latter population originated from an area where intensive agriculture occurs, suggesting that this population developed resistance to ammonium nitrate. Laurila et al. (2002) observed significant differences in body size at metamorphosis and larval period length in Rana temporaria tadpoles. In their study, tadpoles from a northern population did not vary these life history traits with respect to water quantity while tadpoles from a southern population exhibited faster developmental times and smaller body sizes when exposed to quickly

decreasing water volume. In nature, the southern population inhabits ponds which dry out frequently and erratically. The northern population, however, inhabits ponds that are not prone to complete drying. Since populational differences exist, determining lethal and sublethal effects of contaminants based on one or a few populations may not apply to all populations within a species, resulting in conservation efforts that may not be adequate to preserve the whole species.

In a survey of 26 Florida springs, nitrate ( $\text{NO}_3\text{-N}$ ) ranged from 1.10 to 38.00 mg/L (Katz et al. 1999). Total nitrogen in four lakes (Wauberg, Watermelon Pond, Orange and Newnan) in Alachua County, Florida ranged from a minimum of 0.273 to a maximum of 11.500 mg/L (Lakewatch 2001). Nitrate amounts (as  $\text{NO}_3$ ) in Florida ponds typically range from 0.1 to 1 mg/L, although some ponds have nitrate amounts at the 3 mg/L range or higher (USGS 2003). One pond in Gainesville, Florida (Alachua County) where B. terrestris bred in 2003 had 0.0048 mg/L nitrate-nitrogen. The pond where I obtained breeding adults for use in this experiment was also located in Gainesville, Florida. This pond was not situated near agricultural sites and likely exhibited nitrate levels similar to other Florida ponds (< 1 mg/L). The levels used in this experiment were ecologically relevant at 0 mg/L, 0.824 mg/L, 2.472 mg/L, and 4.944 mg/L nitrate-nitrogen. Although Bufo terrestris tadpole body length was not adversely affected by the nitrate concentrations in this study, higher concentrations may produce smaller body lengths since studies that used higher levels found such an effect.

Clutch affected tadpole body length during the course of this experiment. This could be due to genetic variation or nongenetic variation from maternal influences such as egg size and nutrient input in egg yolk (Travis 1980). Travis (1980) observed a

genetic effect on growth rates in Hyla gratiosa by cross-breeding a limited number of males and females and observing the growth rates in offspring. Semlitsch et al. (2000) performed a cross-breeding experiment with Hyla versicolor and concluded that genetic variation in tadpoles, rather than maternal influences, largely affected tadpole tolerance to the insecticide carbaryl. However, a separate, crossbreeding experiment would have to be conducted to conclusively state whether clutch differences in body length and Gosner stage of Bufo terrestris are due to genetic effects.

Tadpoles from Clutch 2 typically had smaller body lengths than tadpoles from either Clutch 1 or 3 until about Day 24 in the control and 5 mg/L treatment, Days 18-22 and Day 28 for 15 mg/L, and Day 22 for 30 mg/L . A potential explanation for Clutch 2 tadpoles having longer body lengths later in the experiment could be the decrease in body length during metamorphic climax, Gosner Stages 42-46 (Emerson 1986). Since tadpoles in Clutches 1 and 3 were ahead in developmental stages they also had smaller body lengths when in Gosner Stages 42-46 (metamorphic climax). Tadpoles from Clutch 2 typically had slightly slower development than tadpoles from Clutches 1 and 3, although clutch did not have a statistically significant effect on time to metamorphosis.

Since density and competition were not factors, longer larval periods did not appear to translate into smaller body sizes at metamorphosis. At the beginning of the experiment, all tadpoles from Clutch 2 were smaller than tadpoles from Clutches 1 and 3. At the end of the experiment, Clutch 2 tadpoles in the control group achieved larger body lengths than Clutch 1 or 3 tadpoles (Fig. 3-2). In the 5 mg/L and 30 mg/L treatments, Clutch 2 tadpoles were still smaller than Clutch 1 or 3 tadpoles at the end of the experiment, although the difference was not as large as it was at the onset of the

experiment (Figs. 3-3 and 3-6). At the end of the experiment, Clutch 2 tadpoles in the 15 mg/L treatment level were larger than Clutch 3 tadpoles. However, they were still smaller than Clutch 1 tadpoles although the size difference was not as great as at the onset of the experiment (Fig. 3-5). Clutch 2 tadpoles also had slightly longer larval periods although this was not statistically significant. Individuals with longer larval periods spend more time in a stable environment and maximize growth at the risk of increased mortality from predation and desiccation thereby achieving a larger body size at metamorphosis (Alford and Harris 1988; Hecnar 1995). Although most Clutch 2 tadpoles were not larger than Clutch 1 or 3 tadpoles, their body lengths did increase during the course of the experiment such that their final lengths showed much less disparity among Clutches 1 and 3 as compared to the beginning of the experiment.

One tadpole from the 15 mg/L treatment (2.472 mg nitrate/L) had a grossly swollen and transparent body. Hecnar (1995) noted many tadpoles with a similar condition when he exposed them to chronic levels (2.5, 5, and 10 mg/L) of nitrate-nitrogen and suggested that nitrate produced the deformity by affecting tadpole osmoregulation. It is possible that nitrate produced the same condition in my experiment. Although only one tadpole exhibited the swelling, it may have been more susceptible to nitrate since individuals vary in their response to contaminants. However, since only one tadpole exhibited this condition in my experiment, it is uncertain what the causative agent may have been.

The fact that the control and 30 mg/L treatments had the highest mortality appear to be a stochastic event unrelated to nitrate concentration. All water parameters among treatments were similar, there was no variability in body length, and all tadpoles were handled equally by the investigators. Length of larval period cannot account for

mortality since control tadpoles required approximately the same amount of time to reach metamorphosis as tadpoles in the 5 and 15 mg/L treatments. Furthermore, since tadpoles in these groups reached metamorphosis at approximately the same time, handling stress from body measurements and staging among these groups would have been fairly consistent and cannot account for mortality differences. A scientific explanation for Clutch 1 displaying the greatest mortality in each treatment is also lacking and may be stochastic as well. Similar studies which staged tadpoles typically did so with much less frequency. Semlitsch and Schmiedehausen (1994) used only one stage measurement (Gosner Stage 42, forelimb emergence) for a measure of developmental rate. Performing fewer tadpole measurements would certainly decrease stress to the animals and, therefore, possibly reduce mortality.

### **Future Research**

Determining effects of varying nitrate levels on all life stages of amphibians inhabiting areas where such contamination occurs is essential to prevent possible population and species declines. Since many amphibian species breed in temporary bodies of water, future experiments should compare effects of nitrates among several populations that inhabit a range of nitrate-contaminated and uncontaminated areas, using levels consistent with those found in the field. Such levels are likely to be much lower than those in current literature. Data on nitrate concentrations in temporary bodies of water are relatively scant, therefore this type of study would add to that body of literature as well by providing such data. Additionally, scant information exists on nitrate effects on developmental rate in amphibians. If nitrate levels decrease developmental rates, amphibians inhabiting temporary ponds may be in danger of dessiccation since metamorphosis may not be completed by the time their pond dries completely. If nitrates

increase developmental rates, it may lead to individuals with smaller body sizes at metamorphosis since a longer larval period typically produces larger metamorphs (Alford and Harris 1988; McDiarmid and Altig 1999). This could reduce fitness if smaller females produce fewer and smaller eggs (Watt and Oldham 1995), ultimately resulting in reduced amphibian populations.

Comparing effects of different types of nitrate would be useful since they produce varying effects on amphibians. Ammonium nitrate, for example, is more toxic to amphibians than sodium nitrate, owing to the ammonium ion and is used extensively in agricultural settings (Schuytema and Nebeker 1999b). Effects on fecundity and fertilization are also lacking in the literature with only one study linking low pH to decreased sperm motility (Schlichter 1981). Decreased sperm motility could negatively affect population growth in habitats heavily contaminated with ammonium nitrate.

Ultimately, many factors work synergistically in the environment to create adverse effects. Without the addition of contaminants, larval mortality is relatively high so that very few individuals reach reproductive ages. With the added stress of contaminants, mortality likely will increase and reduce populations in the future (Semlitsch et al. 2000). Although contaminants may select for resistant individuals in the population, allowing the population to persist and grow, loss of genetic diversity could reduce adaptiveness of the population to future selective pressures. Pesticides, fertilizers, competition, food abundance and water quality all likely have complex roles in developmental and growth rates of larval amphibians. It is necessary to understand the impact of each factor independently and interdependently to predict the future of amphibian populations and create realistic conservation goals.

More detailed studies comparing contaminant effects on species with different life history characteristics need to be undertaken as well. Bufo terrestris exhibits synchronous hatching and metamorphosis. Studies have shown that high density and low food abundance can affect development and growth in other species by producing smaller metamorphs (Smith-Gill and Berven 1979; Wilbur and Collins 1973). Nitrate has been shown to affect body length, larval period length, and feeding activity in other species as well (Hecnar 1995; Marco et al. 1997; Oldham et al. 1997; Rouse et al. 1999). It may be reasonable, therefore, to conclude that nitrate contamination may differentially affect species with varying life history characteristics such as population density and time to metamorphosis, producing more drastic effects in species that breed synchronously in ephemeral bodies of water compared to species with asynchronous breeding in permanent bodies of water. Since temperature can greatly affect the length of the larval period, a laboratory experiment using tadpoles of the same species with varying nitrate concentrations and three temperature regimes may be useful in determining how larval period may interact with contaminants.

Mesocosms are experimental tank communities that mimic natural temporary pond conditions but provide more control, manipulation, and accurate censusing data than natural ponds (Morin 1983). Mesocosm studies would be particularly useful in determining indirect nitrate effects on tadpoles resulting from algal effects, a major tadpole food source. Mesocosm experiments may more accurately reflect contaminant interactions in nature and provide more insight into complex ecological interactions of contaminants in nature.

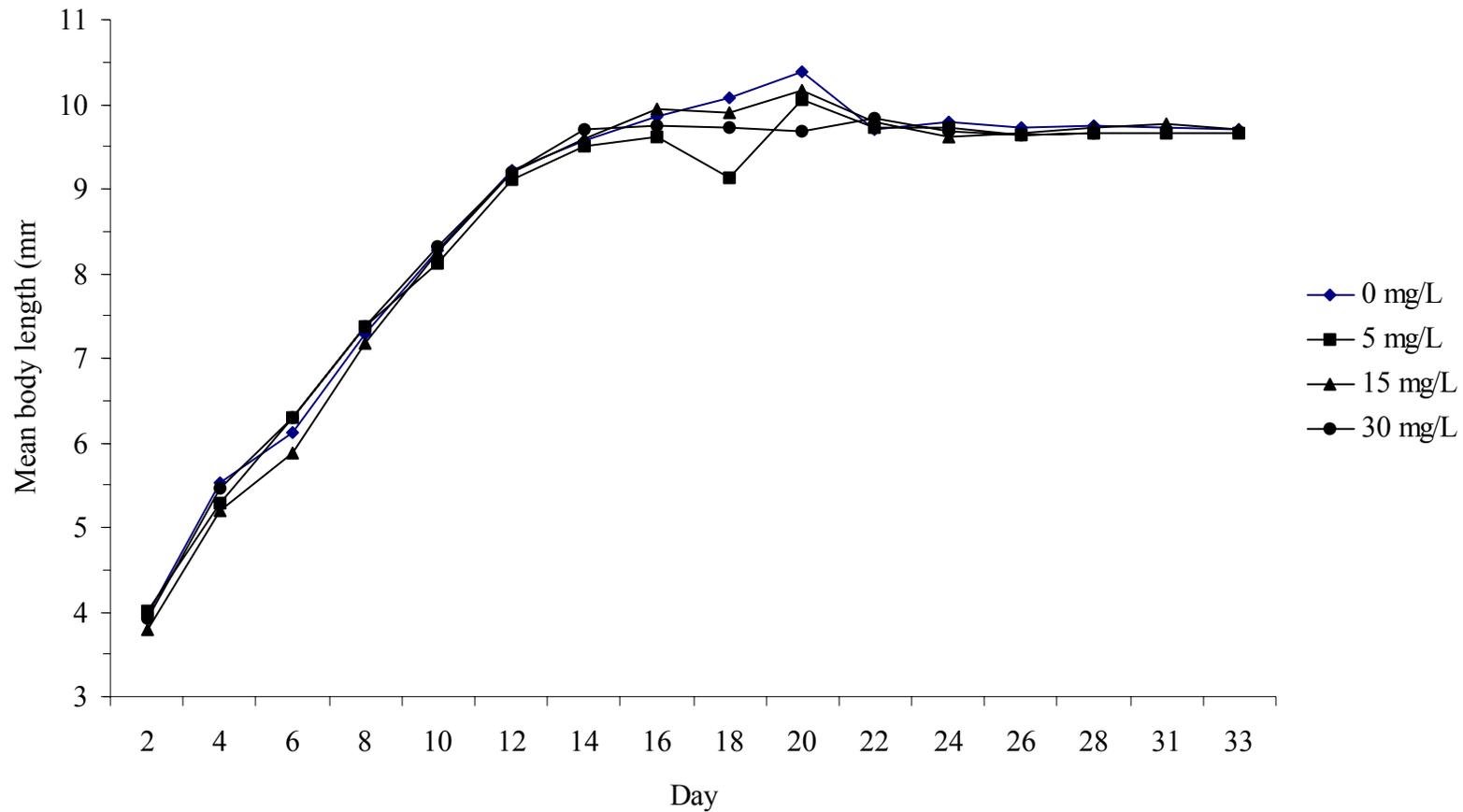


Figure 3-1. Mean tadpole body length by treatment over time. Data were pooled among clutches for each treatment. Data points represent repeated measures of body length throughout the experiment. Day represents time since the start of the experiment when tadpoles were first exposed to nitrate treatments. As tadpoles reached metamorphosis (Gosner Stage 45) in the experiment, their final body length before release was used in calculating the averages for the duration of the experiment. All standard errors ( $\pm$ SE) per day were less than 0.20.

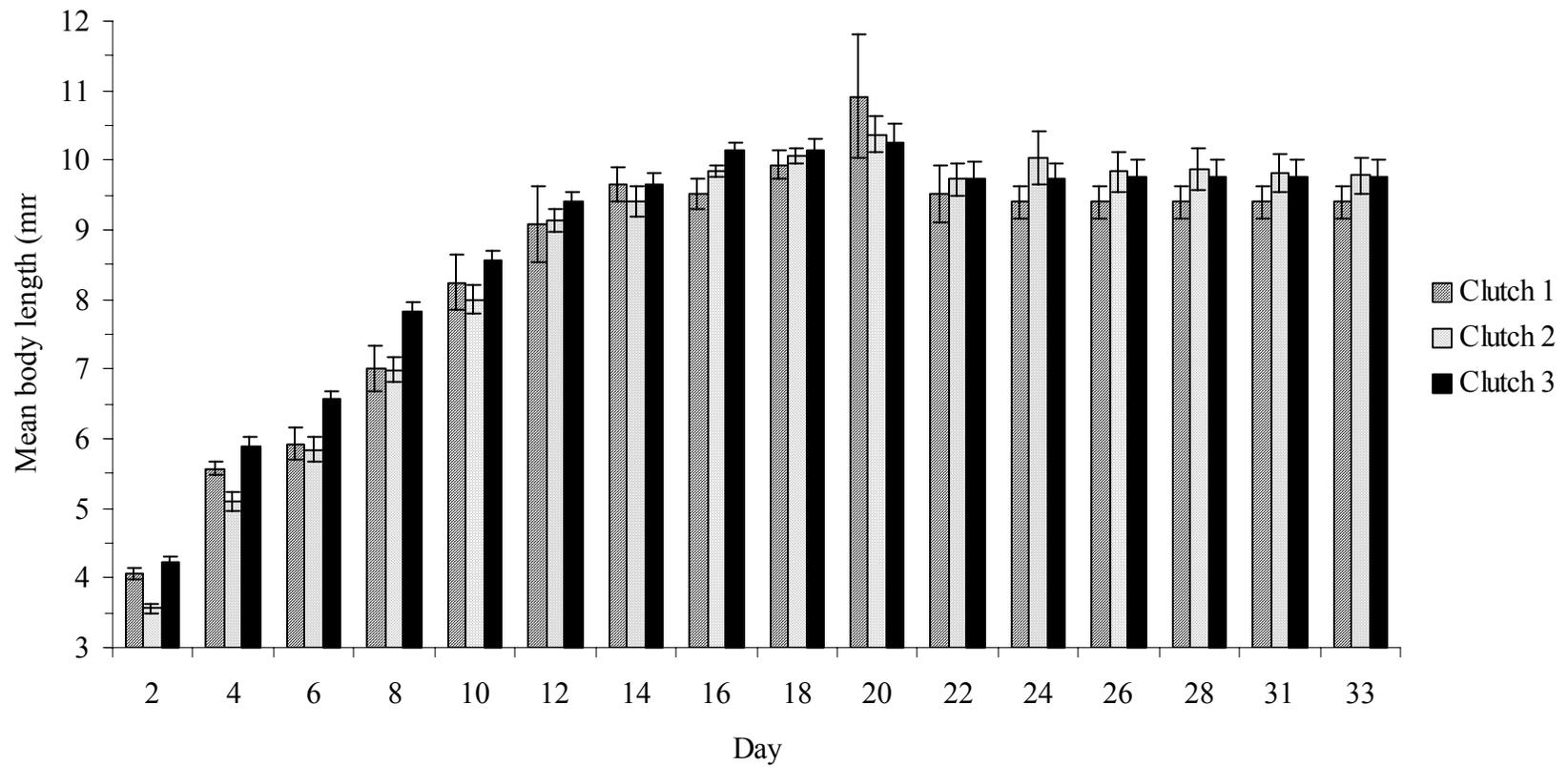


Figure 3-2. Mean body length of tadpoles by clutch in 0 mg/L sodium nitrate treatment. Each clutch represents a separate group of siblings. Day refers to time since the start of the experiment. Error bars represent standard error. Standard error ranged from 0.065 to 0.89.

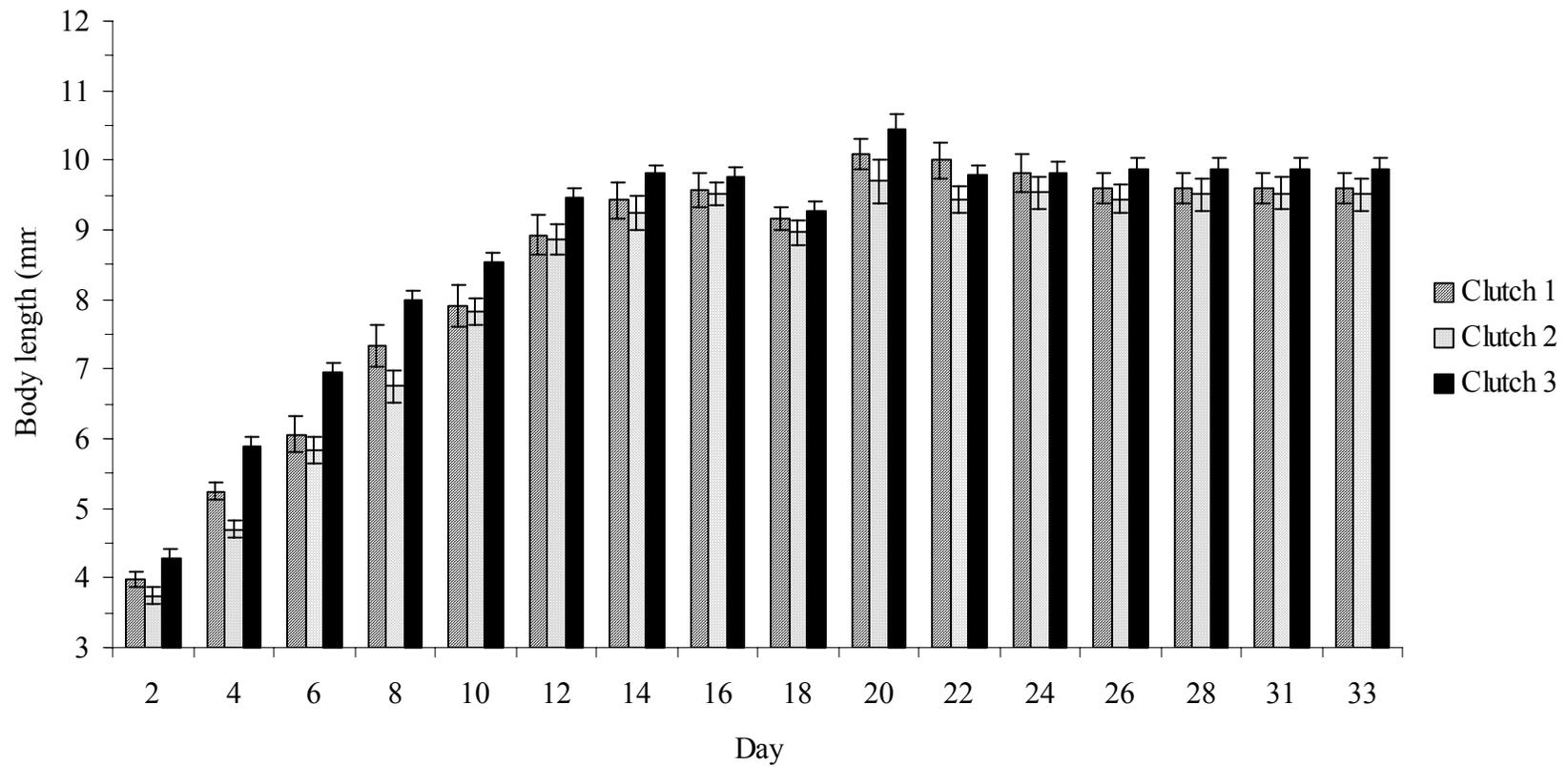


Figure 3-3. Mean body length of tadpoles by clutch in 5 mg/L sodium nitrate treatment. Each clutch represents a separate group of siblings. Day refers to time since the start of the experiment. Error bars represent standard error.

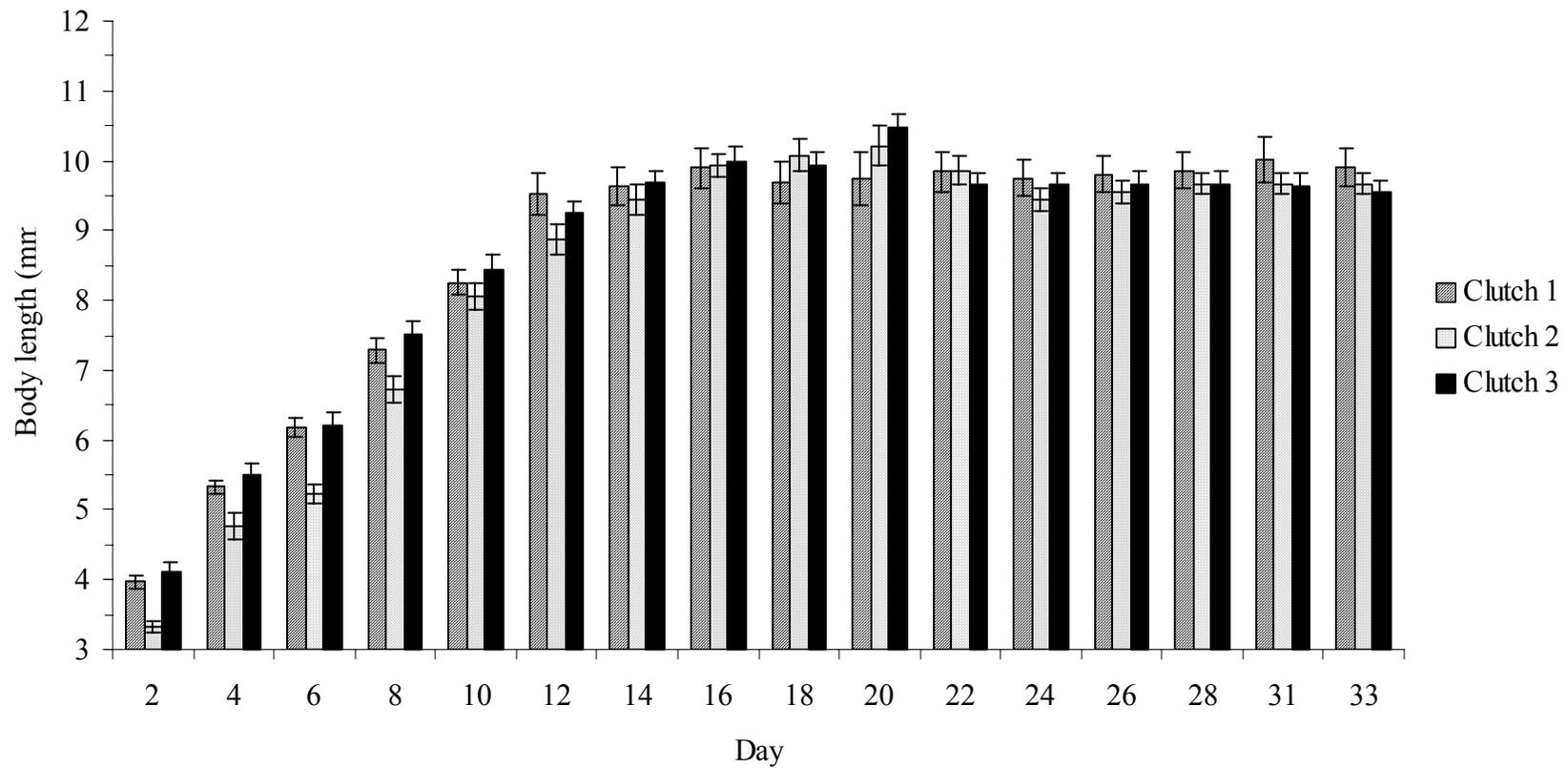


Figure 3-4. Mean body length of tadpoles by clutch in 15 mg/L sodium nitrate treatment. Each clutch represents a separate group of siblings. Day refers to time since the start of the experiment. Error bars represent standard error.

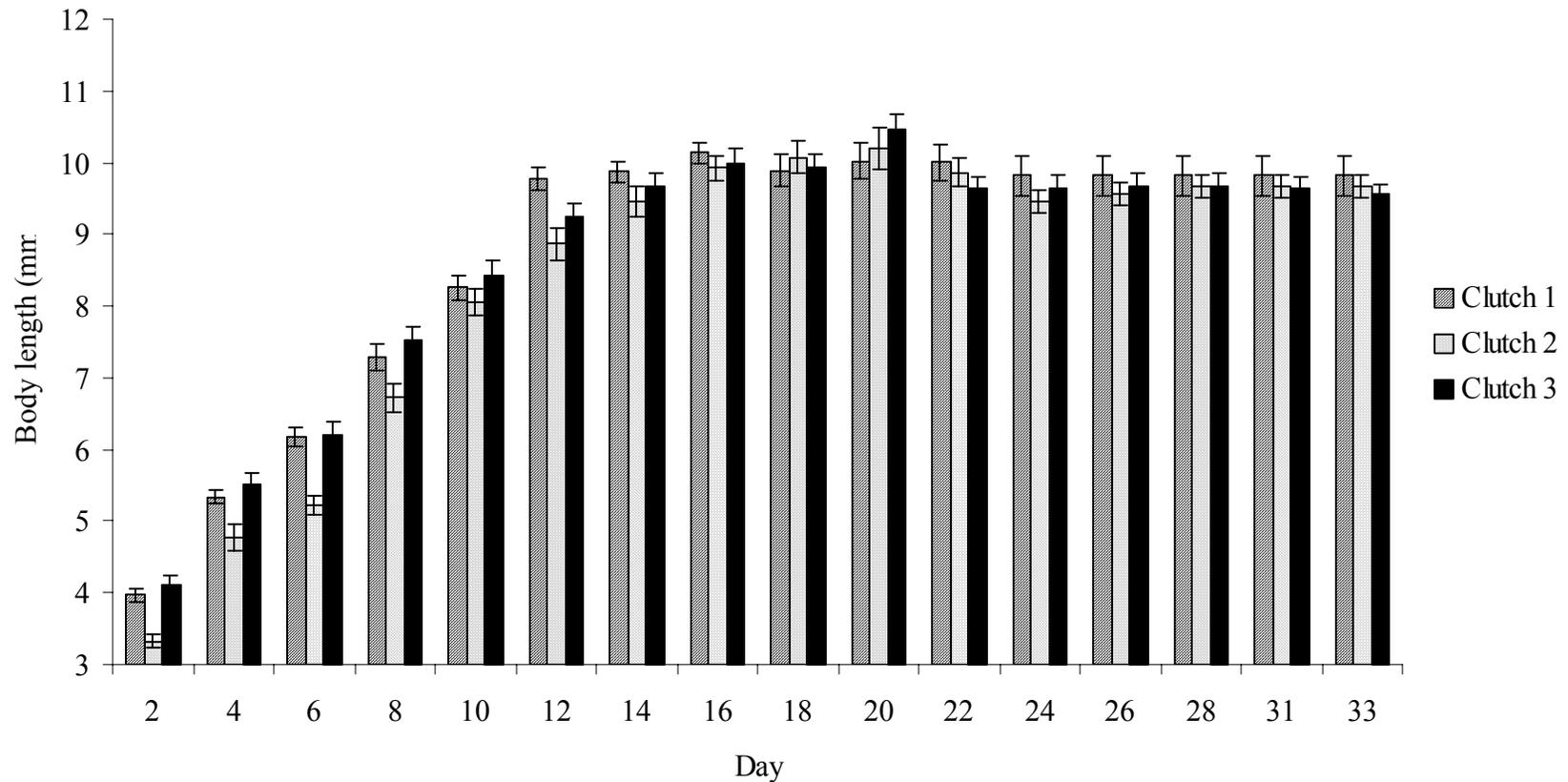


Figure 3-5. Mean tadpole body length of tadpoles by clutch in 15 mg/L NaNO<sub>3</sub> treatment, excluding outlier from Clutch 1. Data were pooled among tadpoles for each clutch and each clutch represents a separate group of siblings. Day refers to time since the start of the experiment. Error bars represent  $\pm$  SE. Elimination of the outlier tadpole produced slightly larger body lengths for Clutch 1 tadpoles on some days.

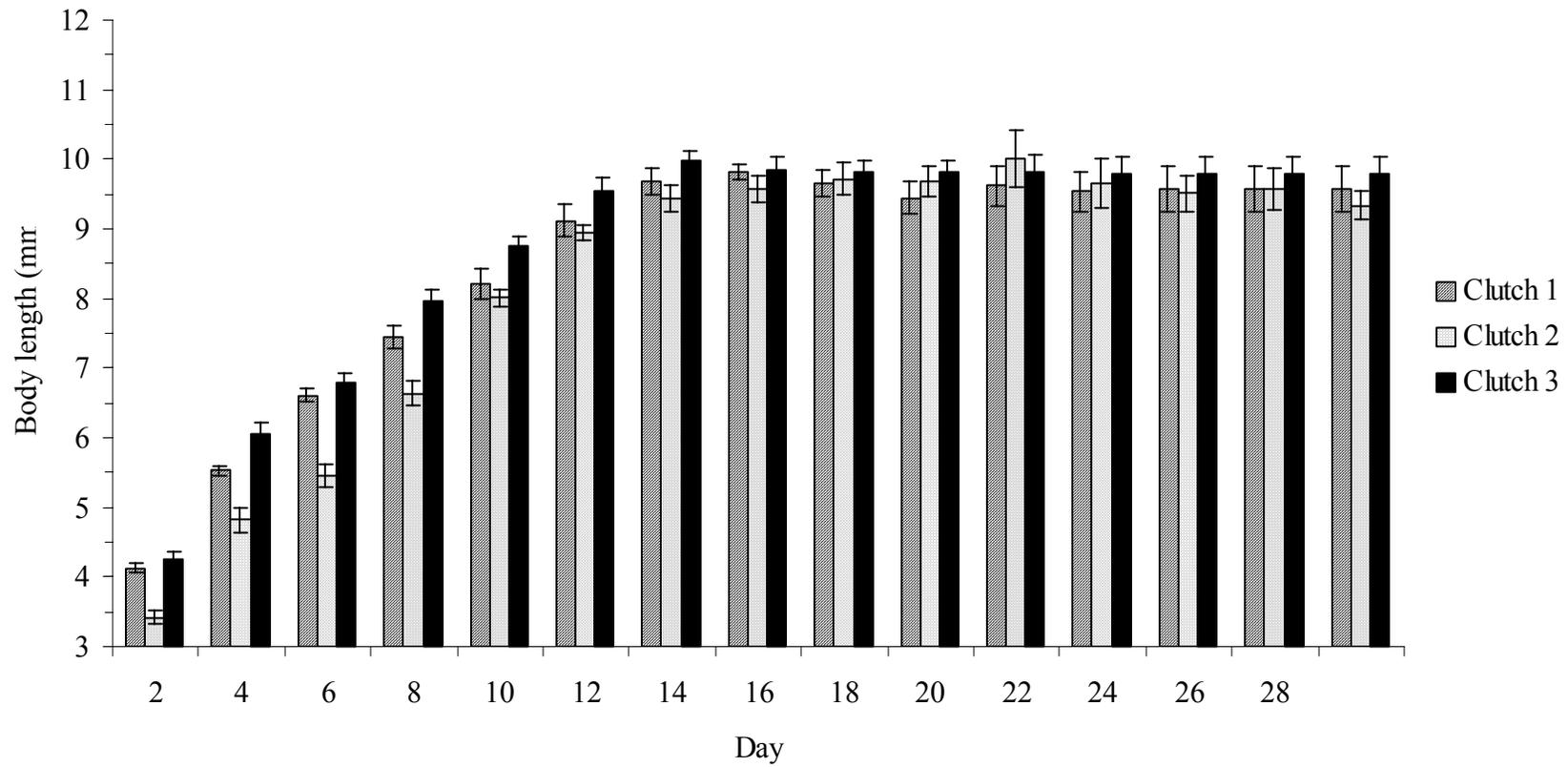


Figure 3-6. Mean body length of tadpoles by clutch in 30 mg/L sodium nitrate treatment. Each clutch represents a separate group of siblings. Day refers to time since the start of the experiment. Error bars represent standard error.

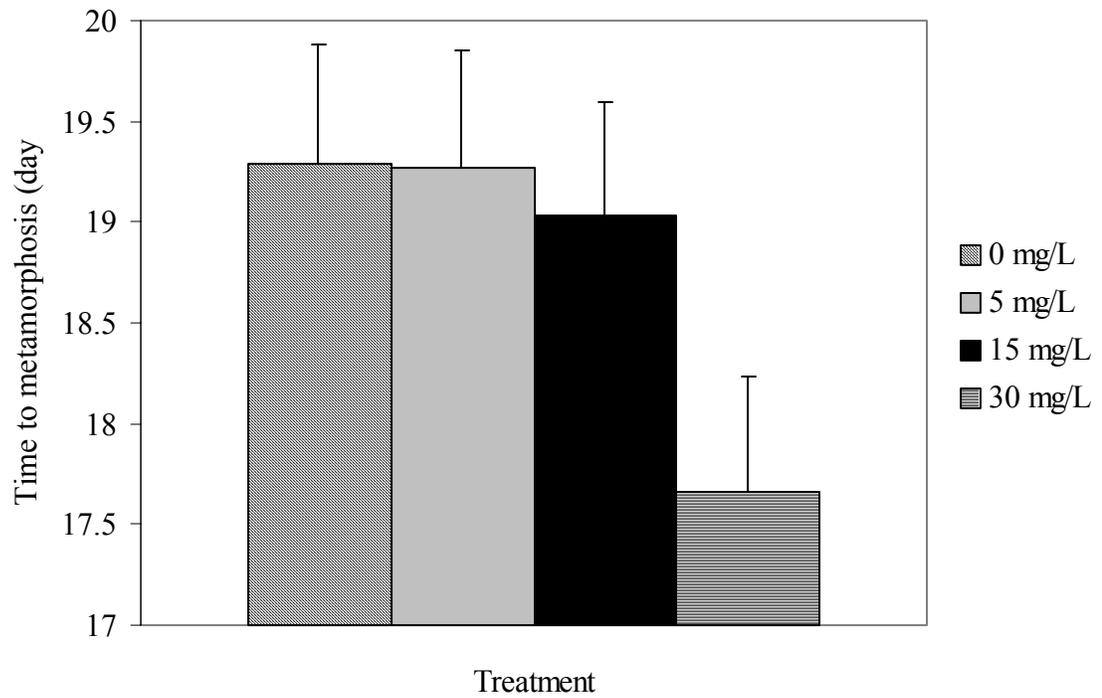


Figure 3-7. Average time to metamorphosis (days) per treatment. Metamorphosis was defined as Gosner Stage 42 (forelimb emergence). Error bars represent standard error (+SE).

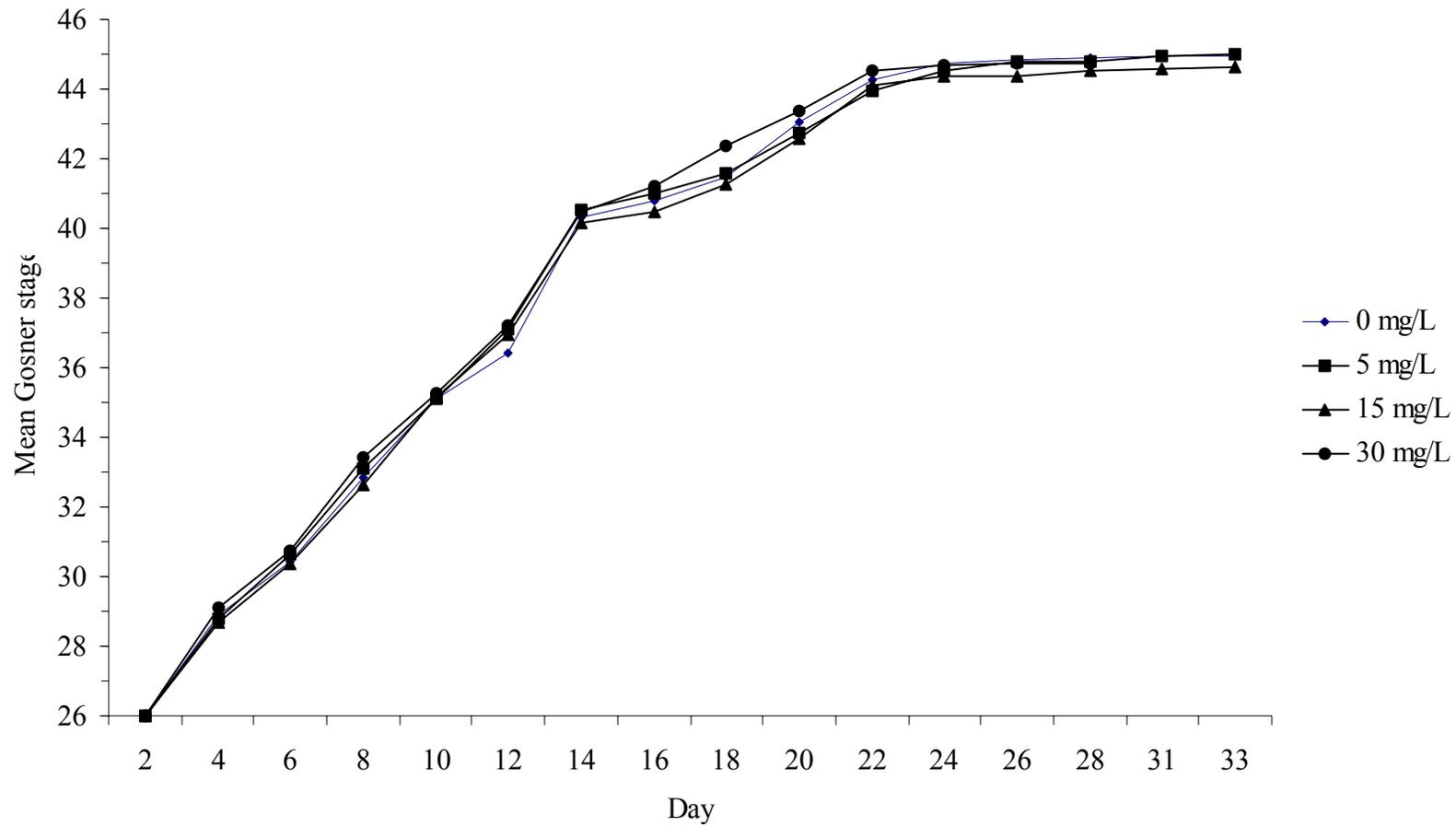


Figure 3-8. Mean tadpole Gosner stage by treatment over time. Data were pooled among clutches for each treatment. Data points represent repeated measures of Gosner stage throughout the experiment. Day represents time since the start of the experiment when tadpoles were first exposed to nitrate treatments. As tadpoles reached metamorphosis (Gosner Stage 45) in the experiment, their final Gosner stage before release was used in calculating the averages for the duration of the experiment. All standard errors ( $\pm$ SE) per day were less than 0.55.

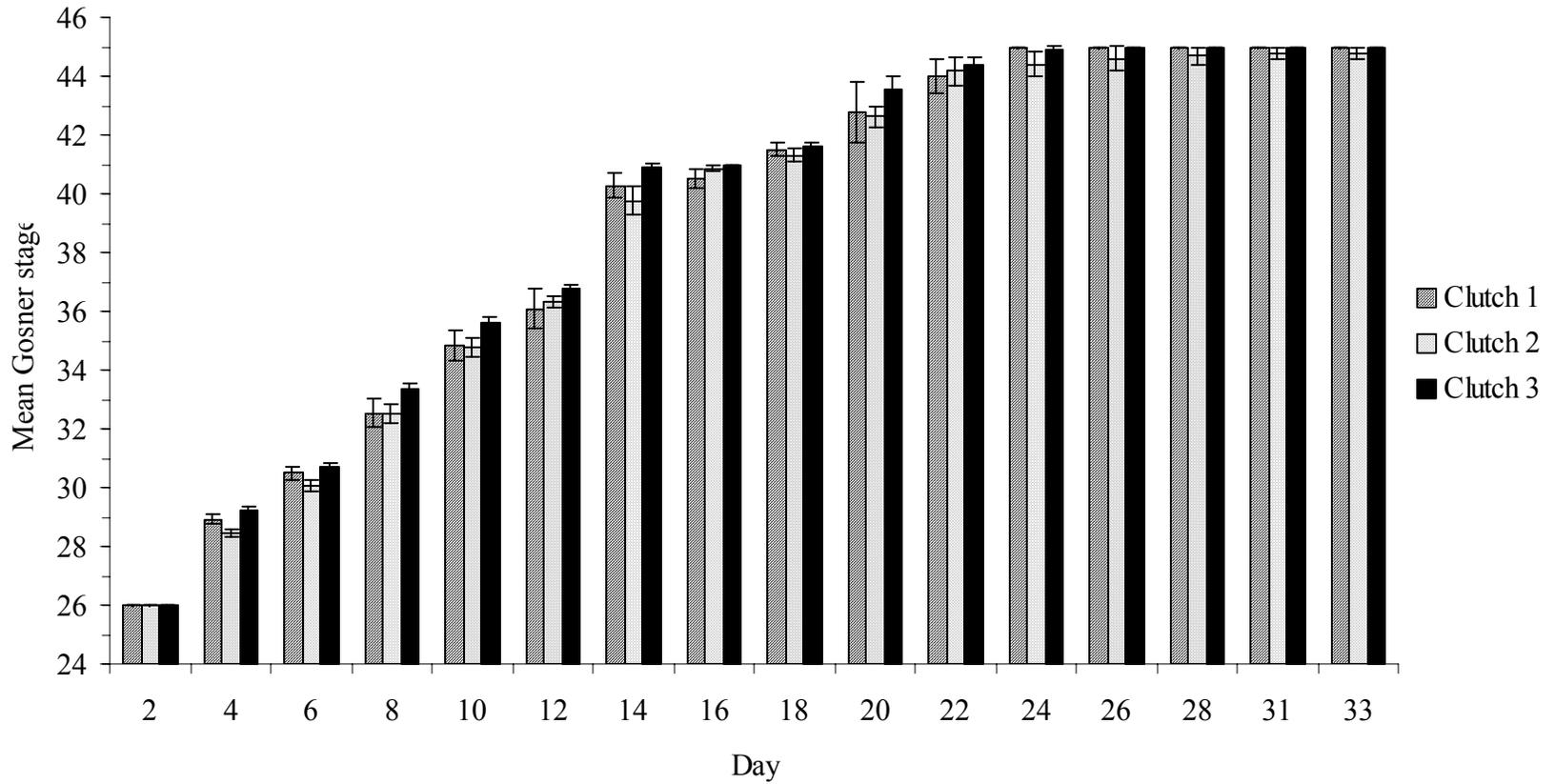


Figure 3-9. Mean Gosner stage of tadpoles by clutch in 0 mg/L sodium nitrate treatment. Each clutch represents a separate group of siblings. Day refers to time since the start of the experiment. Error bars represent standard error.

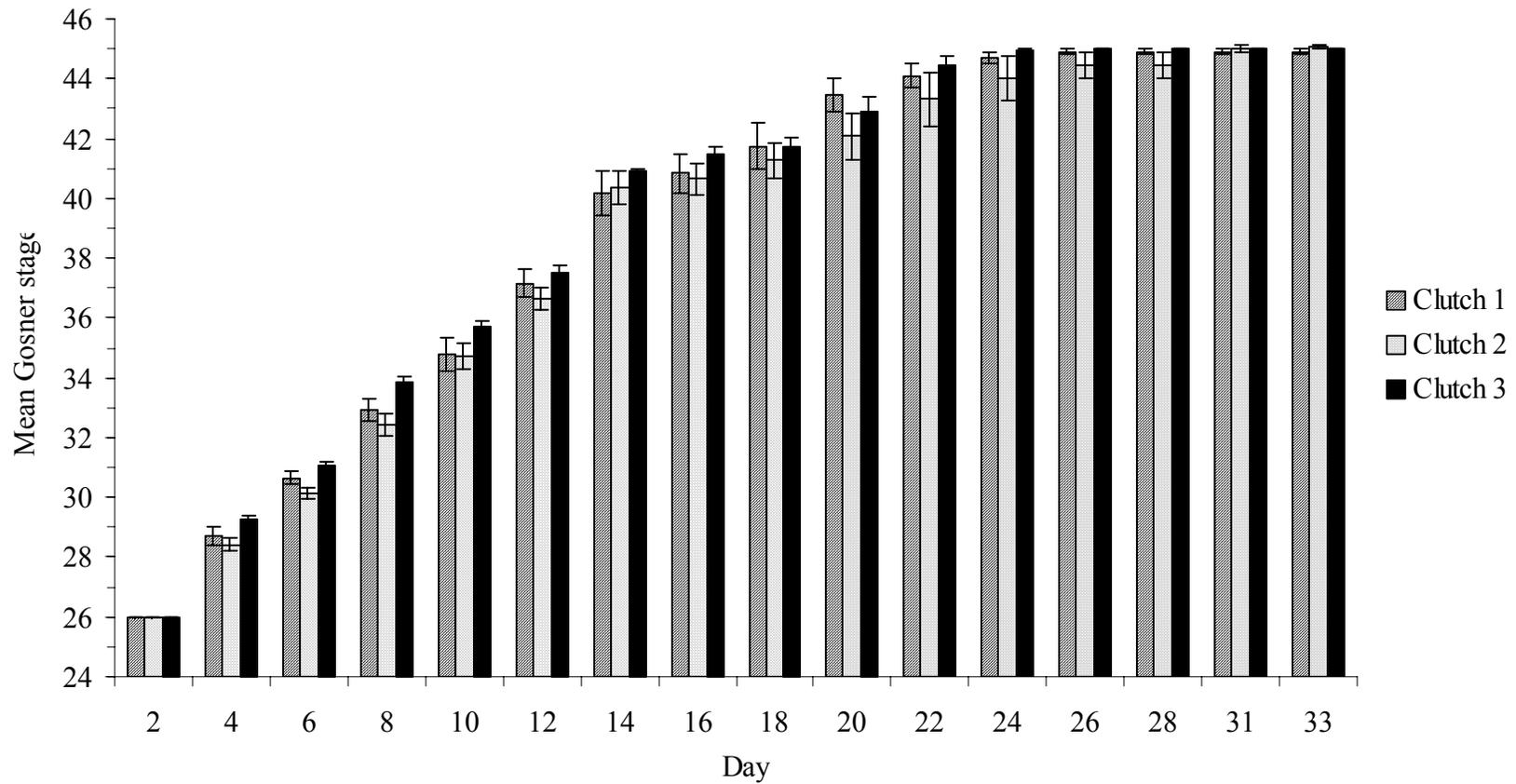


Figure 3-10. Mean Gosner stage of tadpoles by clutch in 5 mg/L sodium nitrate treatment. Each clutch represents a separate group of siblings. Day refers to time since the start of the experiment. Error bars represent standard error.

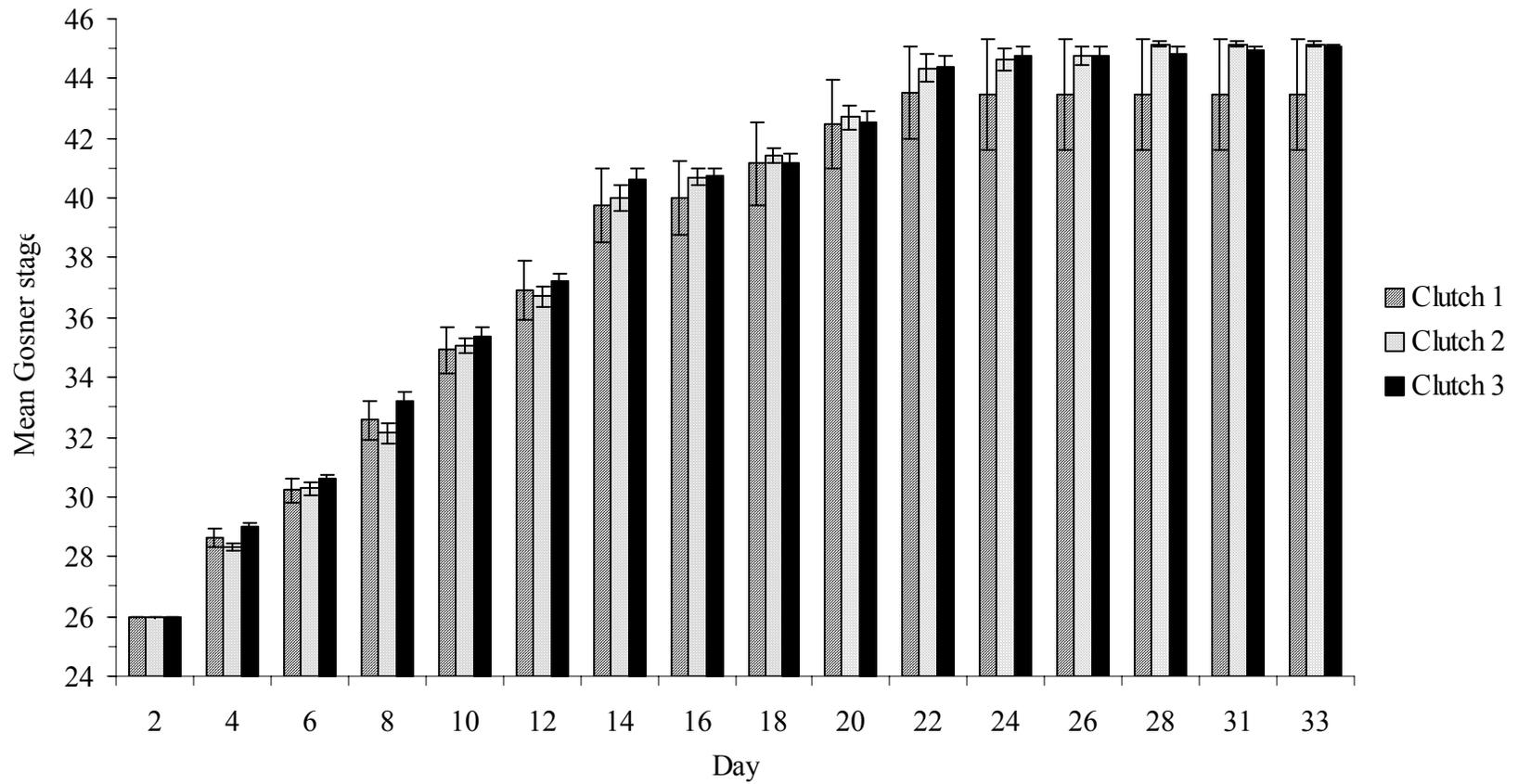


Figure 3-11. Mean Gosner stage of tadpoles by clutch in 15 mg/L sodium nitrate treatment. Each clutch represents a separate group of siblings. Day refers to time since the start of the experiment. Error bars represent standard error.

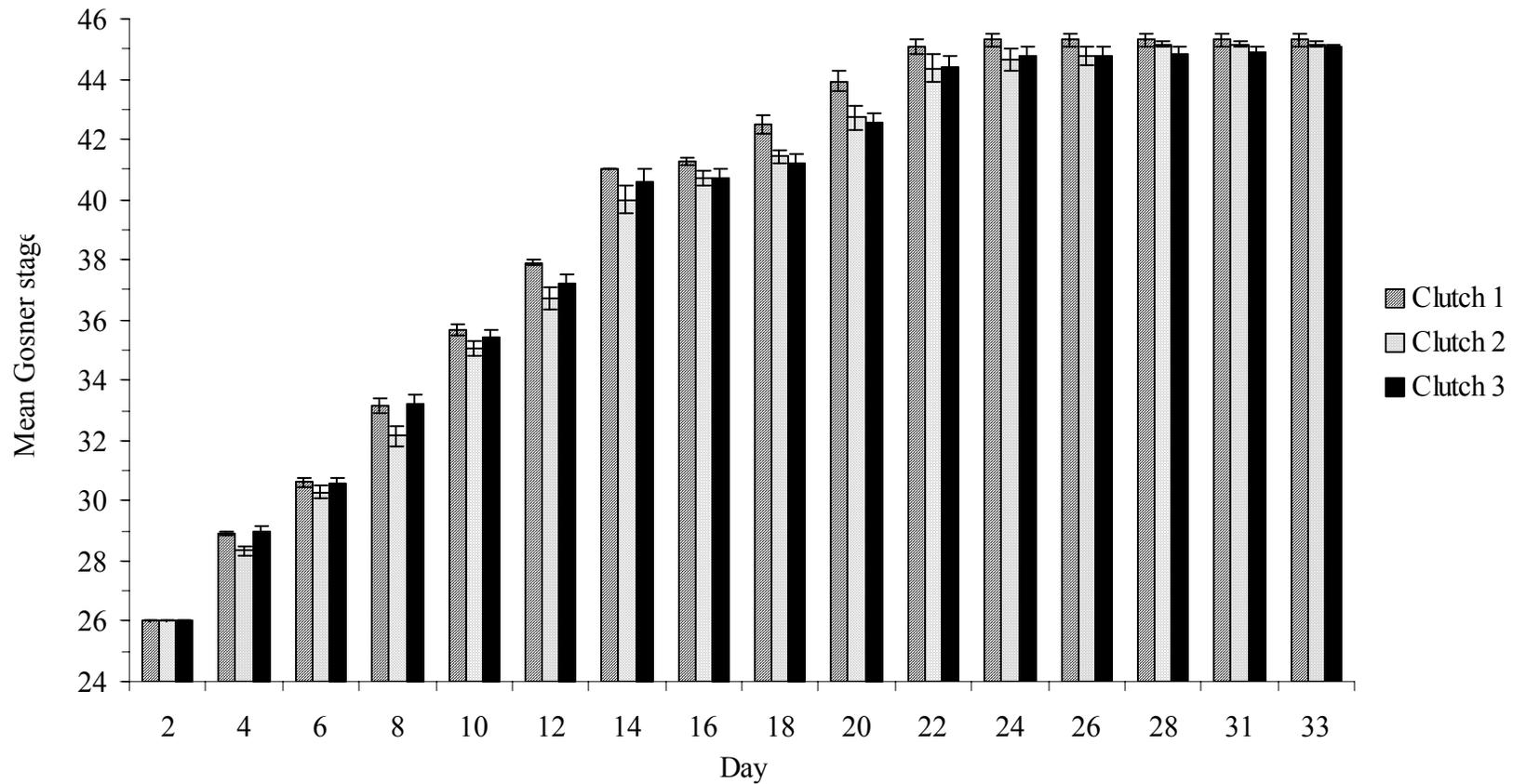


Figure 3-12. Mean Gosner stage of tadpoles by clutch in 15 mg/L sodium nitrate treatment excluding one individual from Clutch 1 that did not develop and remained at Stage 25 throughout the experiment. Elimination of this individual produced a smaller standard error for Clutch 1 than represented in figure 9. Each clutch represents a separate group of siblings. Day refers to time since the start of the experiment. Error bars represent standard error.

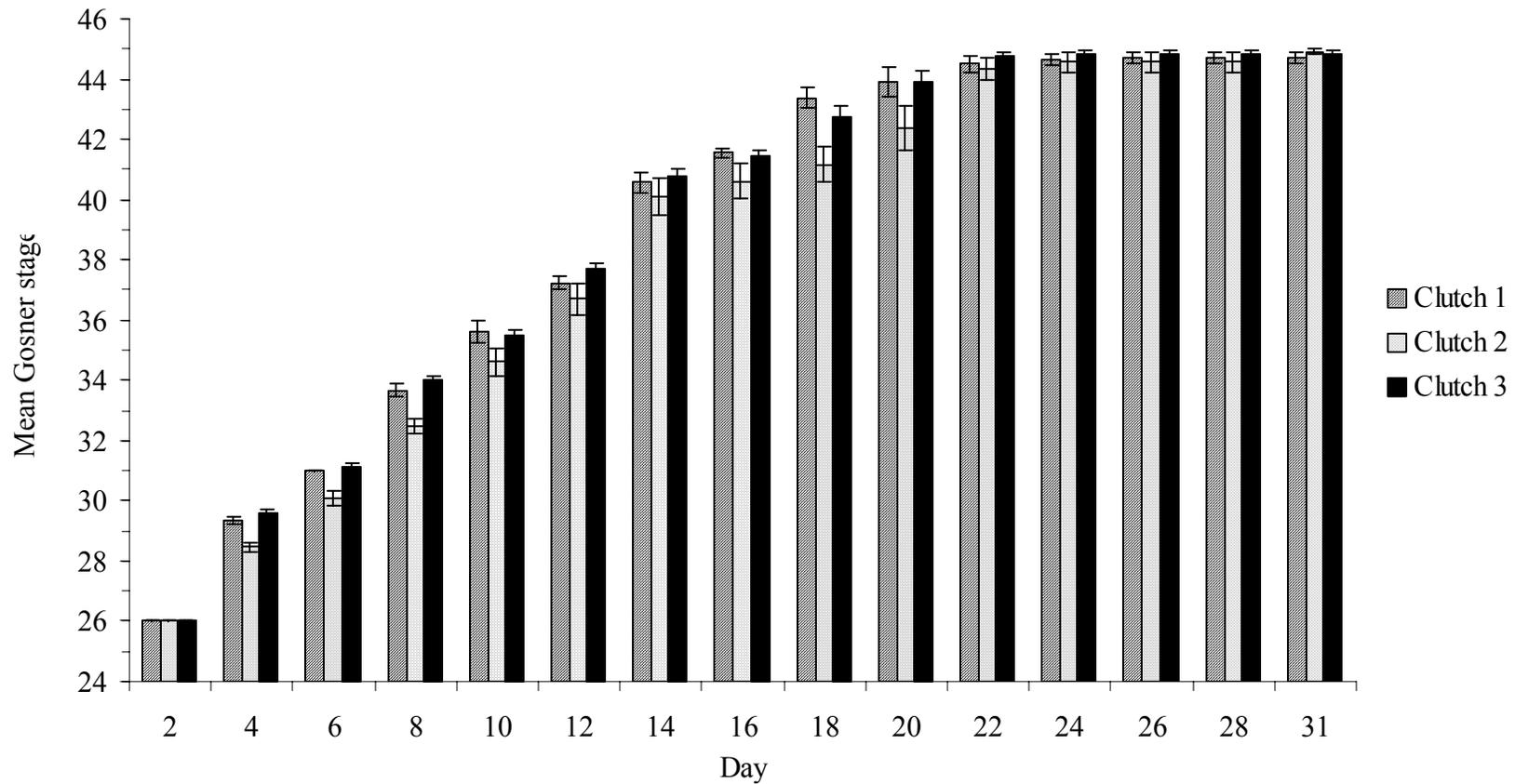


Figure 3-13. Mean Gosner stage of tadpoles by clutch in 30 mg/L sodium nitrate treatment. Each clutch represents a separate group of siblings. Day refers to time since the start of the experiment. Error bars represent standard error.

Table 3-1. Dissolved oxygen samples from random days per treatment and tank within treatment.

Day	Treatment											
	0 mg/L			5 mg/L			15 mg/L			30 mg/L		
	Tank			Tank			Tank			Tank		
	1	2	3	1	2	3	1	2	3	1	2	3
15	8.83	8.81	8.86	8.80	8.80	8.80	8.81	8.80	8.20	8.79	8.78	8.77
16	8.52	8.48	8.50	8.52	8.53	8.54	8.51	8.52	8.54	8.51	8.51	8.51
21	8.42	8.39	8.40	8.40	8.40	8.40	8.43	8.41	8.41	8.32	8.37	8.33
28	*	*	9.80	8.90	*	9.30	*	9.20	*	*	*	9.20
31	*	*	8.10	8.20	*	8.20	*	8.50	*	*	*	*
33	*	*	8.60	*	*	8.40	*	8.40	*	*	*	*

\* Signify tanks that have completed experimentation and are therefore empty. Values are amount of dissolved oxygen in ppm (mg/L). Each value represents one measurement per tank.

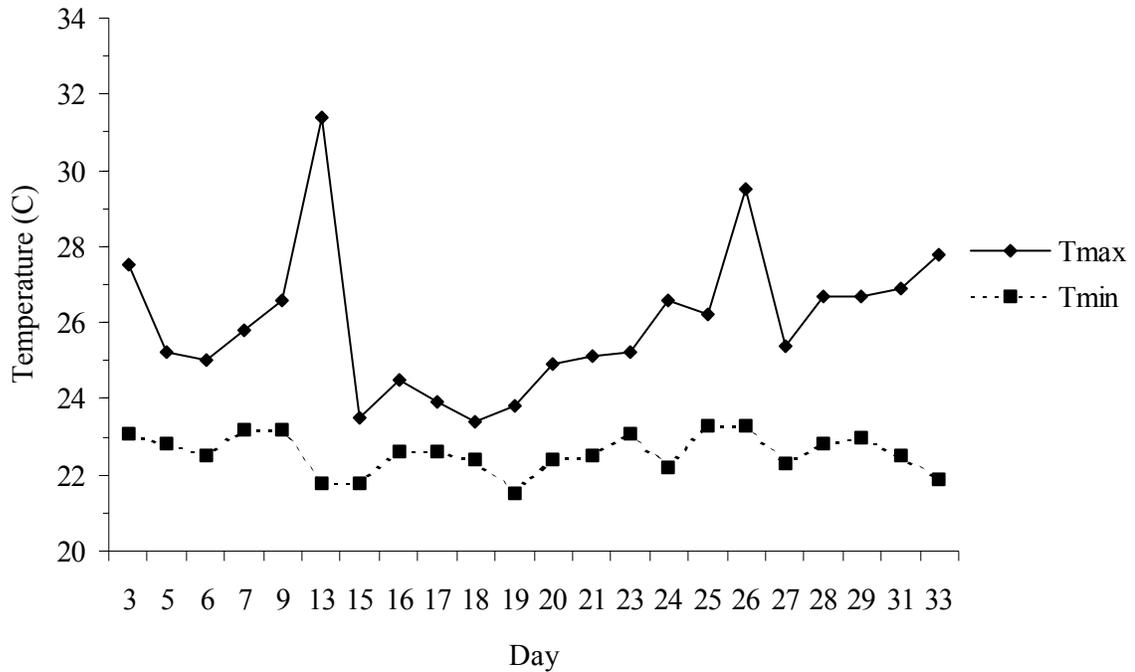


Figure 3-14. Maximum and minimum temperatures (°C) for 0 mg/L sodium nitrate treatment.

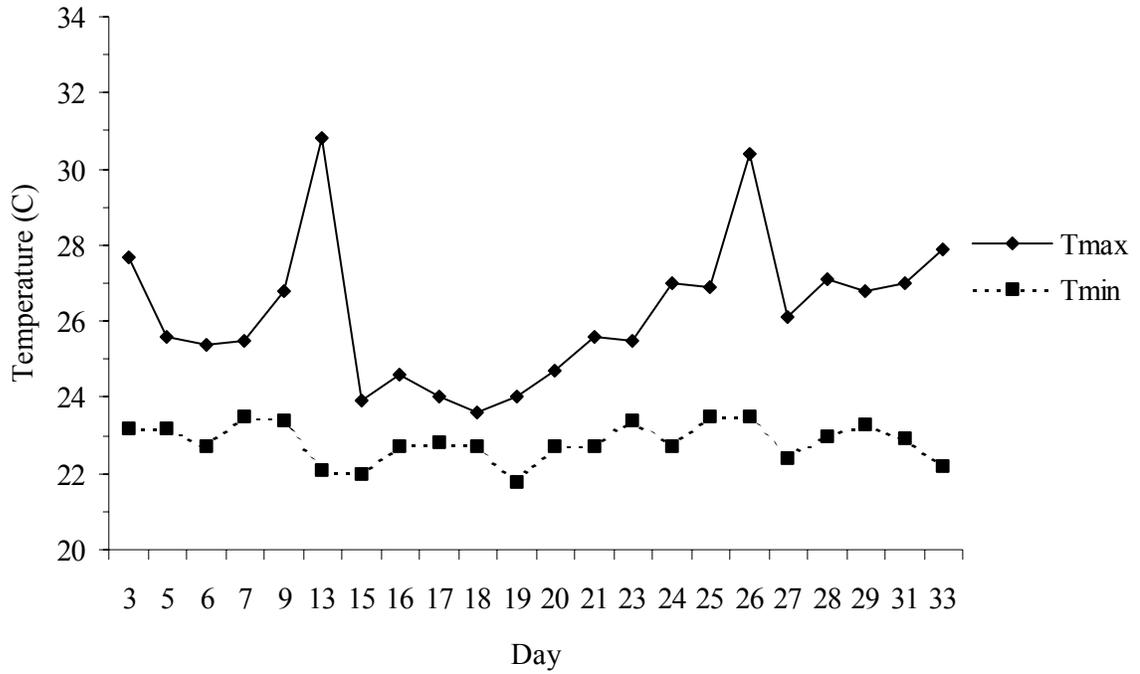


Figure 3-15. Maximum and minimum temperatures (°C) for 5 mg/L sodium nitrate treatment.

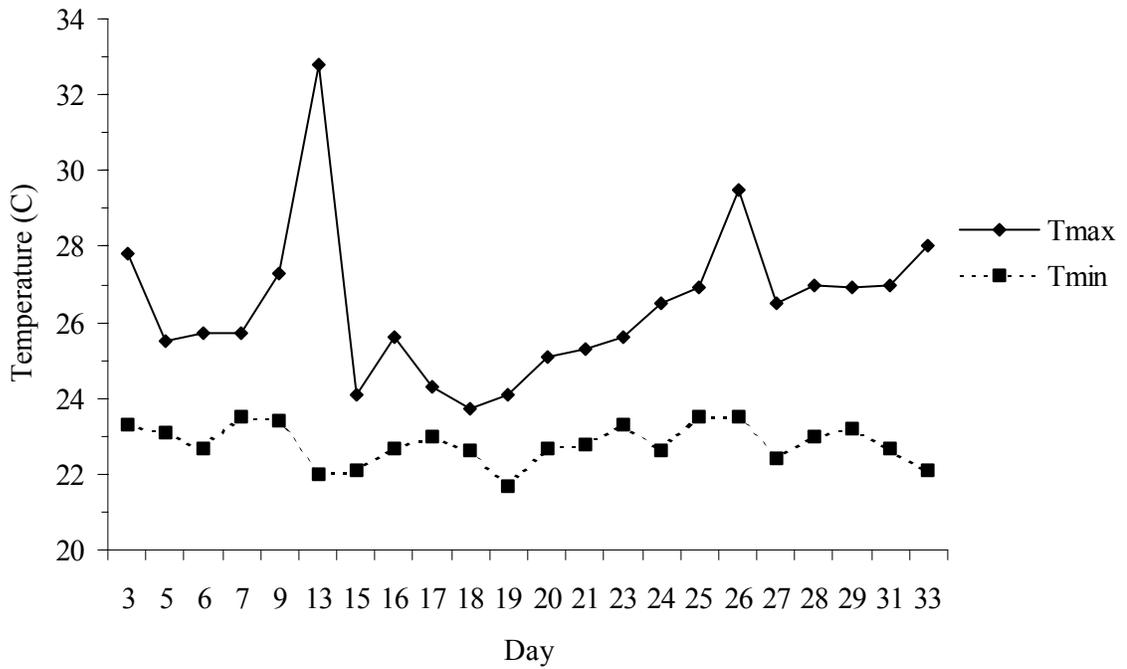


Figure 3-16. Maximum and minimum temperatures (°C) for 15 mg/L sodium nitrate treatment.

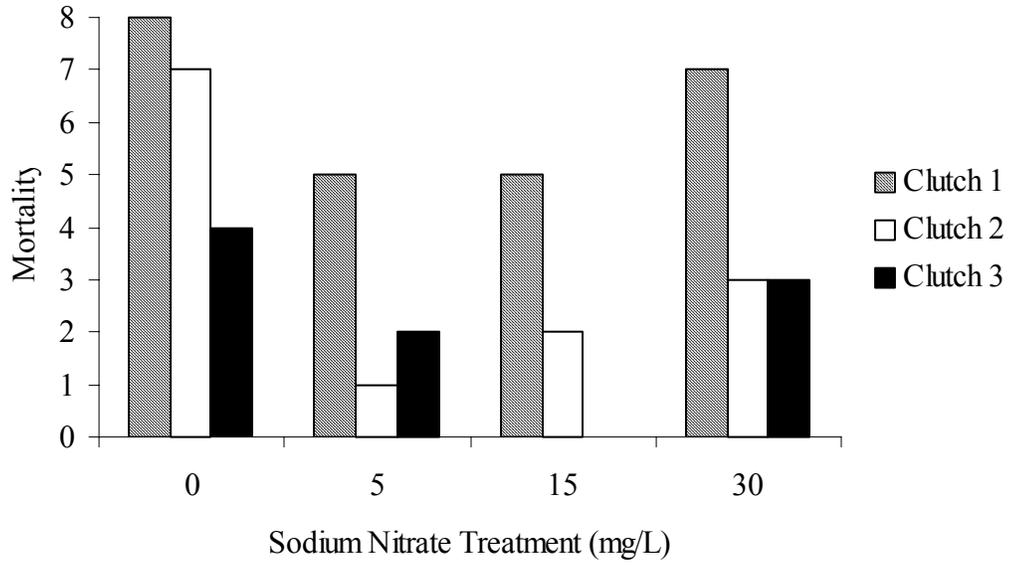


Figure 3-17. Overall mortality in each treatment per clutch.

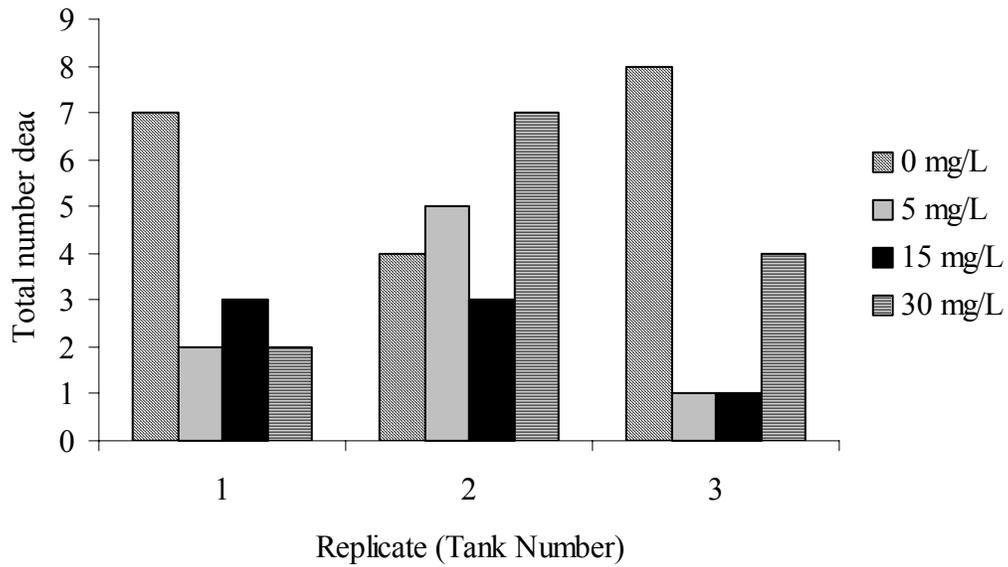


Figure 3-18. Total number of dead tadpoles per replicate (single tank) within treatment

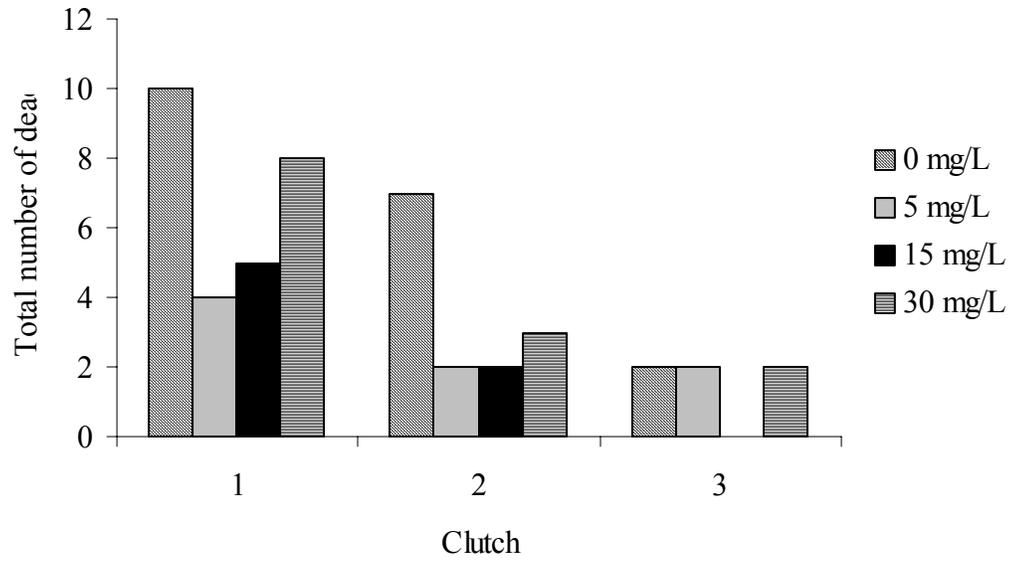


Figure 3-19. Total number of dead tadpoles per clutch within treatment.

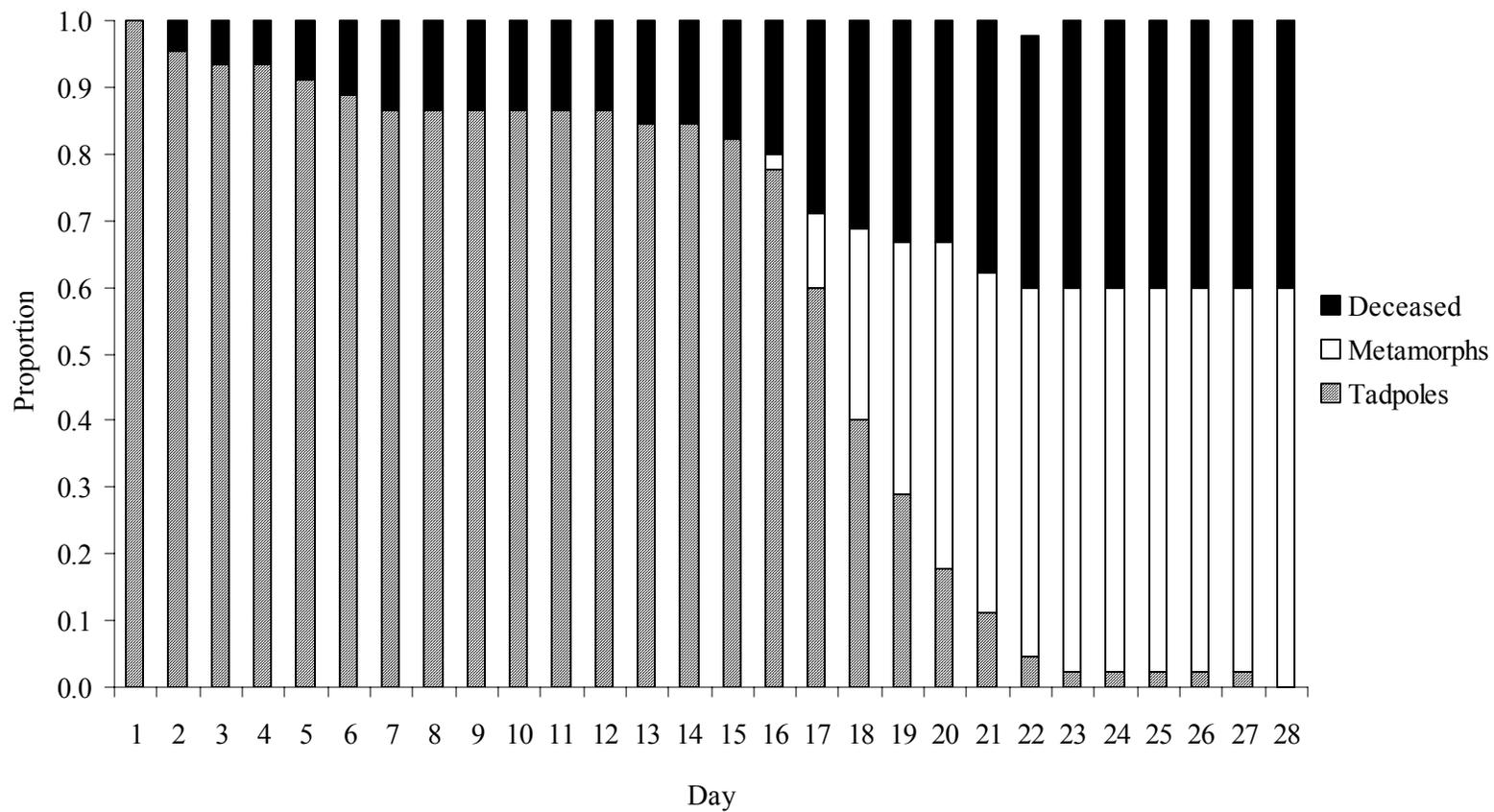


Figure 3-20. Proportion of deceased individuals, metamorphs, and tadpoles for 0 mg/L sodium nitrate treatment.

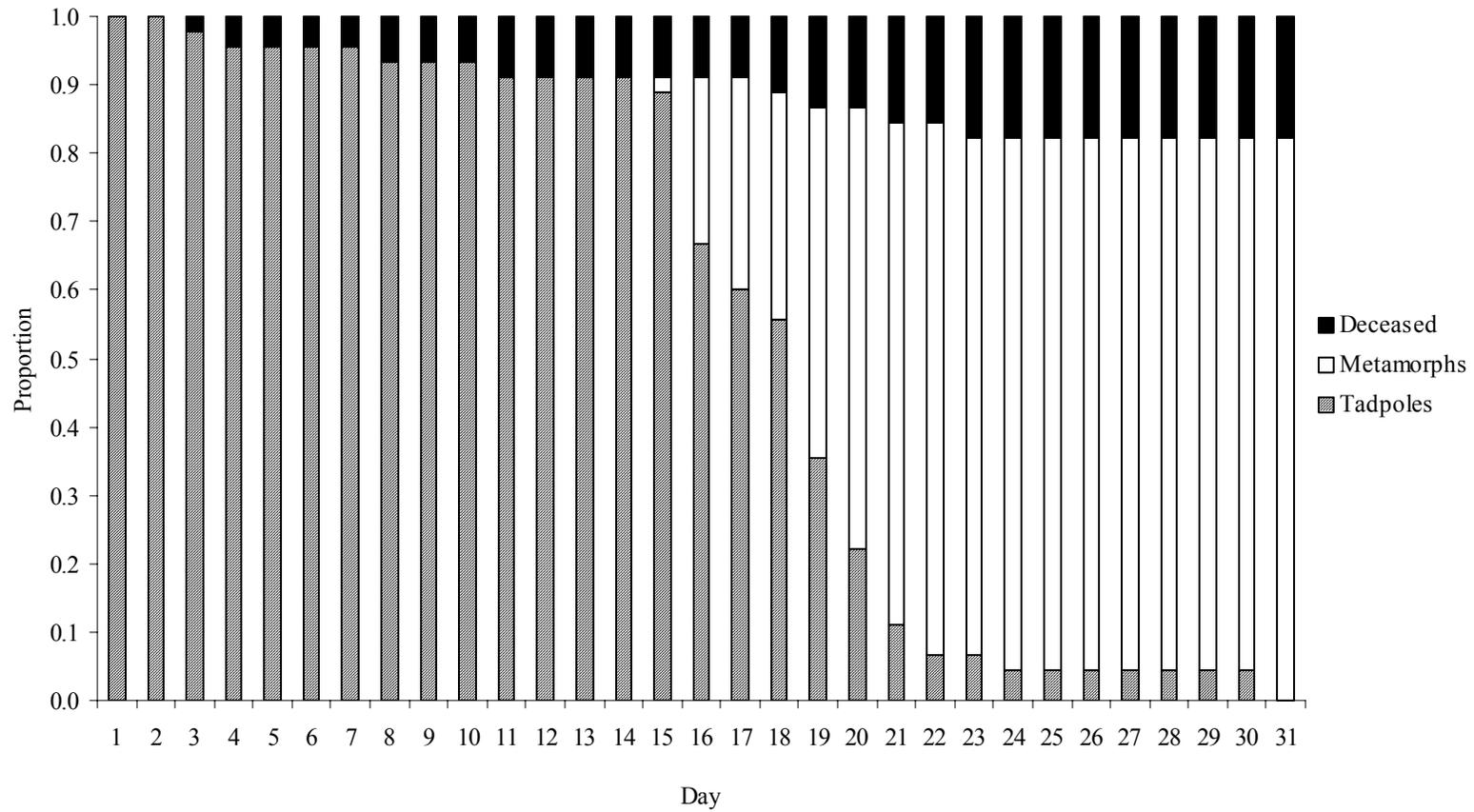


Figure 3-21. Proportion of deceased individuals, metamorphs, and tadpoles for 5 mg/L sodium nitrate treatment.

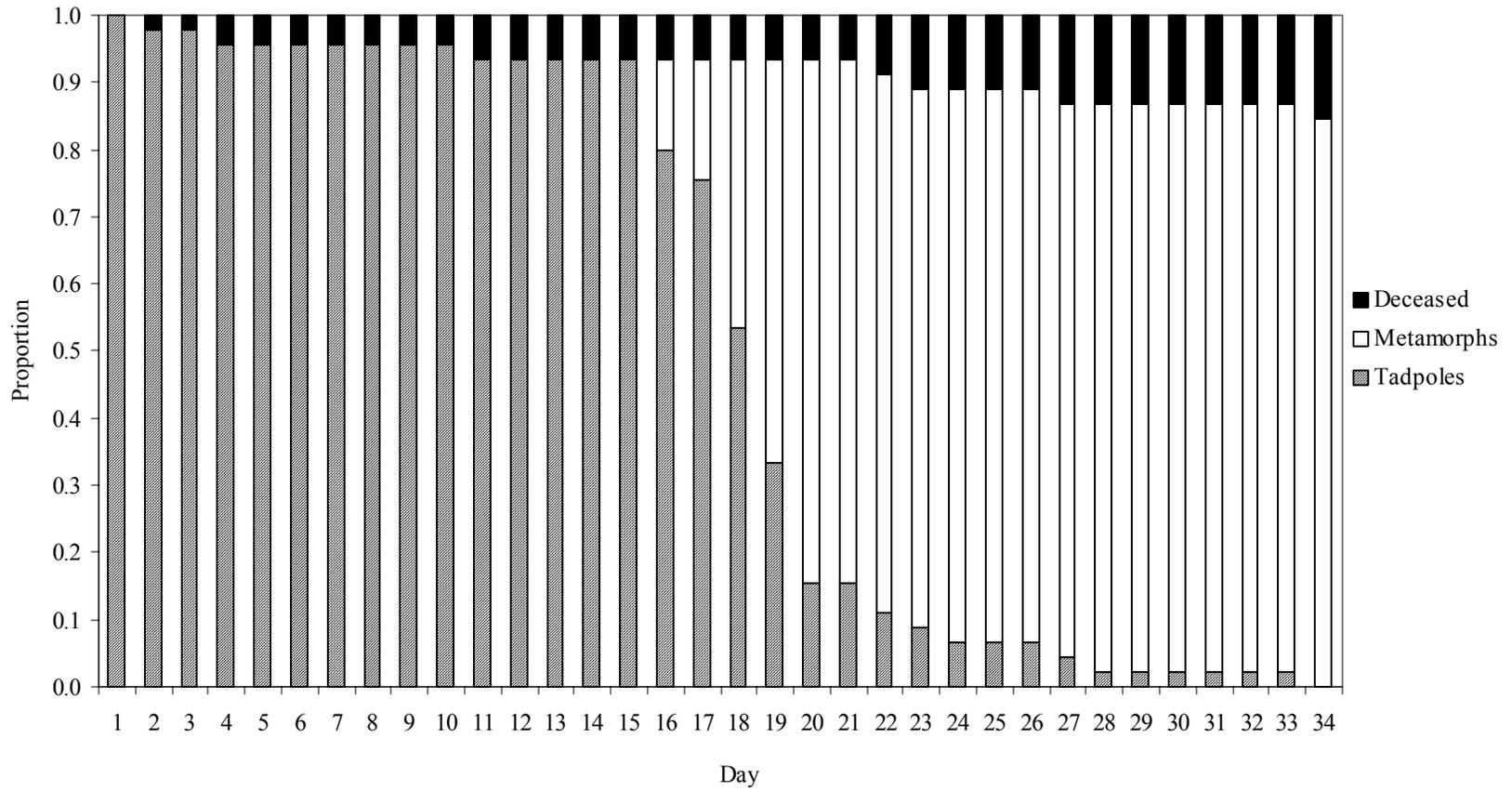


Figure 3-22. Proportion of deceased individuals, metamorphs, and tadpoles for 15 mg/L sodium nitrate treatment.

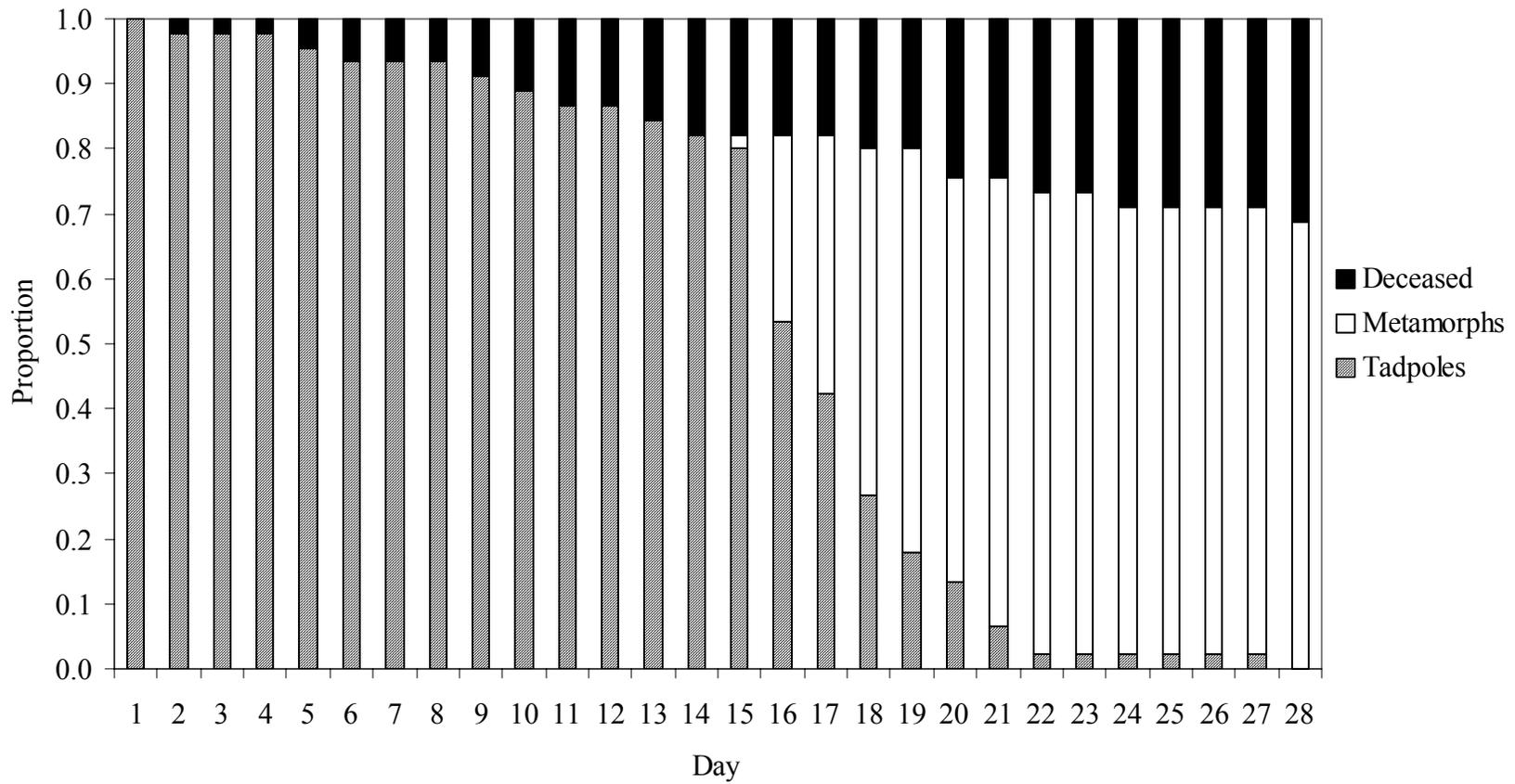


Figure 3-23. Proportion of deceased individuals, metamorphs, and tadpoles for 30 mg/L sodium nitrate treatment.



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