Non-consumptive effects of predatory three-spined sticklebacks (*Gasterosteus aculeatus*) on great crested newt (*Triturus cristatus*) embryos

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Predatory fish have negative impacts on many amphibian populations, often through direct predation on embryos and larvae. The presence of predators during embryonic development may elicit adaptive responses in emerging larvae. This study examined the non-consumptive effects of predatory three-spined sticklebacks (*Gasterosteus aculeatus*) on great crested newt (*Triturus cristatus*) embryos under controlled conditions. Embryos raised in the presence of sticklebacks but in predation-proof enclosures suffered significantly higher mortality compared to control treatments in three independent trials over two years. Overall 26.9% of embryos hatched in stickleback treatments compared to 47.6% from controls. As sticklebacks were treated with fungicide before the experiments, this difference in mortality is unlikely to be due to fungal disease transmission. There were no significant differences in the date, stage of development or size at hatching in larvae raised with and without sticklebacks. The results suggest the potential for negative non-consumptive impacts of predatory sticklebacks on great crested newts during the embryonic stage.

Key words: fish kairomones, mortality, predator cues

INTRODUCTION

The direct predatory effects of fish on amphibians have been well documented in recent years (Beebee, 1996; Hecnar & M'Closkey, 1997; Knapp and Matthews, 2000; Joly et al., 2003). In Europe, introductions of fish to freshwater lakes have been one of the biggest factors contributing to amphibian reproductive failure (Orizaola & Braña, 2006). Many amphibian eggs and larvae are often at particular risk from exposure to predators. Direct predation on eggs and larvae can often drastically reduce numbers and in some cases eliminate whole populations (Bronmark & Edenhamn, 1994; Monello & Wright, 1999). However, predators may also have negative indirect, or non-consumptive, effects on amphibian embryos. Several studies have demonstrated the potential for predatory fish to transfer fungal pathogens, notably *Saprolegnia* species, to amphibian embryos during development (Kiesecker & Blaustein, 1999; Kiesecker et al., 2001). When infected with *Saprolegnia* species some amphibian embryos appear to exhibit increased mortality, resulting in decreased hatching success (Blaustein et al., 1994; Romansic et al., 2007; Fernández-Benéitez et al., 2008). These results suggest the potential for fish to have non-consumptive negative impacts on amphibians at the embryonic stage.

The negative impacts of predation may be reduced if larvae exhibit adaptive responses such as decreased movements (Van Buskirk et al., 1997; Van Buskirk & McCollum, 2000; Relyea, 2002) or morphological plasticity such as developing broader tails (Van Buskirk & Arioli, 2002; Relyea, 2004) when faced with chemical cues from predators. These appear to reduce the effects of predation and thus increase larval survival. There is also increasing evidence to suggest that amphibians can respond to the chemical cues of predators whilst in the embryonic stage. Embryos in immediate danger may exhibit a threat-sensitive response by hatching at an earlier date and stage of development (Saenz et al., 2003; Gomez-Mestre et al., 2008), thus allowing emerging larvae to escape predation risk. If larvae are at greater risk from predation, embryos may hatch at a later date and in a more advanced stage of development (Ireland et al., 2007; Mandrillon & Sagilis, 2009). Larvae hatching from embryos that have developed in the presence of predator cues may also exhibit altered behaviour, such as increased hiding responses (Mathis et al., 2008; Ferrari & Chivers, 2009), or altered morphology such as emerging at a larger size for enhanced swimming (Ireland et al., 2007). These responses decrease the chances of emerging larvae being caught by predators.

Great crested newts (*Triturus cristatus*) breed in permanent or semi-permanent water bodies that occasionally support populations of fish. Their larvae appear particularly susceptible to predation by fish because of their nectonic behaviour (Joly et al., 2001) and whole populations can be eliminated by fish species (Beebee, 1997; Cooke, 1994). Although adults may avoid breeding in fish ponds using chemical cues (Malmgren, 2003), this may only apply to new immigrants (Beebee, 2007). Returning adults, which are highly faithful to breeding ponds, often still breed where fish are present.

Three-spined sticklebacks (*Gasterosteus aculeatus*) are small, predatory, fast-swimming fish that readily consume the larval stages of great crested newts (Oldham et al., 2000). In ponds with sticklebacks, populations of newts will often be decimated (Cooke, 1994; English Nature, 2001; Beebee, 2007). Removal of sticklebacks from ponds can result in recovery of great crested newt populations

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(McLee & Scaife, 1992), demonstrating the potentially negative effects of this species. Although it has been observed that sticklebacks affect newt populations through direct predation, no study has examined the possibility of indirect, or non-consumptive, effects of sticklebacks on embryos before hatching. Only 50% of great crested newt embryos are expected to survive to hatching due to a chromosome abnormality (Horner & MacGregor, 1985). Any negative effects on embryos are therefore likely to have a greater effect on the hatching success of this species. In addition no study has determined whether great crested newt embryos exhibit plasticity in development while in the presence of predators as embryos, which may reduce direct predation on hatching larvae.

This study examined the non-consumptive effects of stickleback predator cues on great crested newt embryonic development under controlled conditions. The main aims were to determine whether great crested newt embryos 1) show any changes in mortality when developing in the presence of predators while protected from consumption, and 2) exhibit plasticity in timing of development and morphology whilst developing in the presence of predatory sticklebacks.

METHODS

Experiments were carried out in three independent trials: early and late season 2008 and late season 2009. Due to cold conditions experienced in the early part of 2009, no early season experiments were carried out. The 2009 experimental trial adopted a different methodology to eliminate the potential for fungal transfer from sticklebacks to embryos.

2008

Ten opaque plastic containers (55 cm L × 45 cm W × 33 cm D) were filled with aged tap water and placed on a laboratory bench at room temperature (20±3°C) and were subject to natural light:dark cycles. Egg strips, consisting of black plastic bags cut into strips approximately 2 cm wide and 50 cm long, were placed into a local great crested newt breeding pond in March 2008. After 48 hours, strips were removed from the pond and the eggs were counted. Eggs were unwrapped to allow the assessment of developmental stage (staging table in Gallien & Bidaud, 1959), but remained on strips and were divided into equal groups. Females may lay several eggs per strip as well as laying on multiple strips (personal observation). Therefore, eggs from different strips were randomly assorted before grouping. Each group, containing approximately fourteen eggs, was placed into a mesh bag (20 × 20 cm; mesh diameter <0.5mm). Each mesh bag was sealed and placed into an individual container, ensuring eggs were submerged. Sticklebacks were placed into alternate containers; the remaining five containers were left as controls. This allowed for five sticklebacks and five control replicates. Sticklebacks were fed every 48 hours using commercial fish flakes (TetraFin®: containing mainly fish derivatives, cereals, algae, molluscs and crustaceans). Care was taken to ensure the fish ate all food immediately. Tanks were not oxygenated since the relatively large volume of water (81 litres per tank) was assumed to contain enough oxygen for the duration of the experiments. Water pH and NO_3^-/NO_2^- analyses were carried out weekly in each container. After two weeks, containers were checked daily for hatched newt embryos.

Four weeks after the start of the experiment all surviving embryos had hatched, and all containers were emptied, cleaned and the experiment repeated with the same procedure in May 2008. Different individual sticklebacks were used and twenty (rather than 14) great crested newt eggs were placed into each container. Embryos were collected from the same breeding pond. This contains over 200 breeding females (personal records based on capture–recapture studies), so it is unlikely that individual embryos used for different trials were produced by the same females.

2009

In the 2009 experiments, embryos were left wrapped to reduce fungal transfer (e.g. *Saprolegnia* species) from fish to embryos. To avoid the adding of fish food to experimental tanks, sticklebacks were left in containers for 48 hours before being returned to a separate oxygenated feeding tank. Different sticklebacks were then placed in the experimental tanks. Sticklebacks in the feeding tank were also treated with the fungicide silver proteinate (0.0151% w/w), by pipetting 1 ml of fluid for each 9 l of water in the tank.

Data analysis

The percentage of embryos hatching in each trial was calculated, along with their means. A two-way ANOVA was applied to arcsine-square root transformed proportion data to test for a difference in hatching rates across treatments and trials. The mean number of days to hatching, stage of development and total length at hatching were compared using two-way ANOVA.

RESULTS

Embryo survival

In all trials, a significantly smaller proportion of embryos hatched from stickleback treatments compared to control treatments (ANOVA, $F_{1,24}$=13.18, P=0.0013). Overall, 26.9% of embryos hatched in stickleback treatments compared to 47.6% from controls (Fig. 1). There was no significant interaction in hatching between trials (ANOVA, $F_{2,24}$=0.55, P=0.59), indicating no effects of treatment on hatching rates. In control containers from all trials, the mean percentage hatching from each tank was close to the expected 50% (taking the chromosome abnormality of crested newts into account). In stickleback treatments, the mean proportions of embryos hatching from each trial were 23%, 24% and 33%, respectively.

Date, stage of development and total length at hatching

There was no significant difference in the mean number of days to hatching (ANOVA, $F_{1,24}$=1.33, P=0.26), the stage of development ($F_{1,24}$=1.08, P=0.31), or the total length of larvae at hatching ($F_{1,24}$=1.34, P=0.26) between

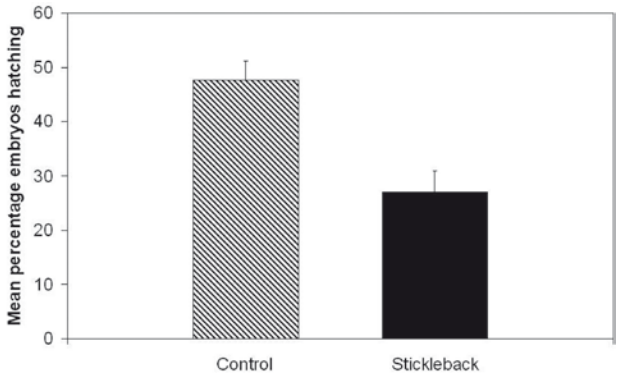

Fig. 1. Mean percentage great crested newt (*Triturus cristatus*) embryos hatching in both stickleback and control treatments in three independent trials over two years. Error bars denote standard error.

stickleback treatments and controls in any of the three trials. The mean number of days to hatching was 13.99±1.10 days and 15.34±0.58 days in stickleback and control treatments, and the mean stages of development at hatching were 37.95±0.19 and 38.13±0.26, respectively. The total length at hatching was 10.79±0.15 mm in the stickleback treatments, compared to 10.89±0.13 mm in the controls. There was no significant interaction between mean date, stage of development and total length at hatching between trials (ANOVA: $F_{2,24}$=1.66, P=0.21; $F_{2,24}$=1.44, P=0.34; $F_{2,24}$=2.65, P=0.09, respectively).

Water analysis revealed a constant pH of 7.0 in all containers during all trials. Water NO_3^- and NO_2^- never exceeded 25mg/l and 1 mg/l respectively in all containers.

DISCUSSION

Embryo survival

The results from this study show increased mortality of great crested newt embryos when developing in the presence of predatory stickleback cues. Great crested newts developing naturally lose 50% of embryos to a chromosome abnormality (Horner & MacGregor, 1985). In the presence of three-spined sticklebacks, survival was only between 23 and 33%. Decreased survival may have deleterious impacts on potential recruitment, especially if the negative consumptive impacts of sticklebacks continue to operate on the larvae of this species. These results also suggest that predators may influence their prey through mechanisms other than direct predation. Possible causes of the observed increase in mortality are mechanical stress, low oxygen levels, altered water nitrate or pH, transfer of fungal infections, increased metabolic demands and predator-induced stress. Mechanical stress was unlikely to have been a problem since sticklebacks were only able to get within approximately 5 cm of embryos. Low dissolved oxygen may lead to abnormalities or asphyxiation, especially in artificial conditions (Seymour & Bradford, 1995; Olivier & Moon, 2010), but low oxygen levels are unlikely to have affected embryo survival since sticklebacks are small fish with low oxygen demands and were only placed in rather large tanks for short time periods. Nitrogen ions have mixed effects on amphibians (Ortiz et al., 2004; Griffis-Kyle & Ritchie, 2007; Meredith & Whiteman, 2008), although many studies show deformities and increased mortality in the presence of these ions, and excretory products from fish may increase nitrogen ions to toxic levels (Mandrillon & Saglio, 2007). However, NO_3^- and NO_2^- levels did not exceed local pond water levels of 25g/L and 1mg/L respectively, and it is therefore unlikely that they are responsible for the observed increase in mortality. Altered pH is known to cause deleterious effects on amphibian embryos and larvae (e.g. Beattie et al., 1991; Griffiths, 1993). However, the pH of the water in all containers remained consistently at 7.0 throughout the experiments. The transfer of pathogens, especially the water mould *Saprolegnia*, from predatory fish to amphibian embryos has been demonstrated in a number of cases (Kiesecker et al., 2001; Kiesecker & Blaustein, 1999; Fernández-Benéitez et al., 2008). The transmission of fungal infections from sticklebacks to developing great crested newt embryos was, however, unlikely, since sticklebacks were treated with antifungal fluid in the last two trials, and there were no significant differences in survival between trials with and without treatment.

Fish are known to produce chemicals, or kairomones, from their skin surface (Ślusarczyk, 1999; Lass & Bittner, 2002; Lass et al., 2005). These are produced either from the skin's mucus cells or from bacteria that inhabit the skin surface (Ringelberg & Van Gool, 1998, in Weber, 2003). The effects of fish kairomones have been found to induce adaptive responses in amphibians (Relyea & Mills, 2001; Relyea, 2003). It is possible that stickleback kairomones affect newt embryos by inducing a stress or metabolic response that may affect immune function. This may lead to increased susceptibility to water-borne pathogens, including bacterial and fungal diseases. Few experimental studies have demonstrated increased amphibian embryo mortality induced by fish kairomones. In a study of damselfly larvae *Lestes viridis*, Slos et al. (2009) found increased mortality in the presence of fish kairomones due to a fight or fright response leading to oxidative stress. Stress or any induced metabolic response triggered by fish kairomones could also be a reason for the increased mortality of great crested newt embryos. Such a response may impair immune function, leading to increase in infection and subsequent mortality. However, my experiments did not test this directly, and the actual causes of mortality require further investigation.

Date, stage of development and total length at hatching

No significant difference in the date, stage or length at hatching of great crested newt larvae in the presence of sticklebacks compared to control treatments were ob-

served. Although embryos of several amphibian species show plasticity in development and morphology when developing in the presence of predators (Mandrillon & Saglio, 2007; Mathis et al., 2008; Ferrari & Chivers, 2009), such plasticity may only occur in certain species and under particular circumstances. For example, embryos of the common frog *Rana temporaria* appear to alter development only when faced with multiple stressors (Mandrillon & Sagilis, 2009). Plasticity may vary depending on the predator and prey species involved. Embryos of the red-eyed tree frog *Agalychnis callidryas* respond to predators by emerging from eggs at an earlier stage of development, while those of the gliding tree frog *A. spurrelli* remain motionless. In addition, Ireland et al. (2007) found that tadpoles of the green frog *Rana clamitans* hatched at a smaller size and earlier stage of development when in the presence of leeches *Nephelopsis obscura*, and a larger size and later stage of development when raised in presence of dragonfly nymphs *Aeshna canadensis* (Mathis et al., 2008). Such phenotypic plasticity in development and morphology is costly (DeWitt et al., 1998), and larvae must experience relatively high predation risk to make plasticity worthwhile (Teplitsky et al., 2003; see also review in Bernard, 2004). Van Buskirk (2002) found that larval *R. temporaria* did not alter their morphology when raised with predatory dragonfly nymphs *A. cyanea*, because the costs of altering body form did not outweigh predation risk. Similarly, Anderson & Petranka (2003) found that neither *R. sylvatica* nor *Ambystoma maculatum* showed any alterations in hatching time or stage of development when raised in the presence of predatory dragonfly nymphs. Again, costs of plasticity outweighing benefits gained were suggested as reasons for lack of response. In my study on great crested newts, the degree of predation risk may be too low relative to the potential costs associated with altering hatching time or morphology; embryos may have needed to face a greater predation risk to elicit a response. Further studies will be required to determine if great crested newt embryos exhibit any form of adaptive plasticity.

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