

**EARLY LIFE SENSORY ABILITY – VENTILATORY
RESPONSES OF THORNBARK RAY EMBRYOS (*RAJA
CLAVATA*) TO PREDATOR-TYPE ELECTRIC FIELDS**

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ABSTRACT

Predator avoidance is fundamental for survival and it can be particularly challenging for prey animals if physical movement away from a predatory threat is restricted. Many sharks and rays begin life within an egg capsule that is attached to the sea bed. This sedentary life stage is especially vulnerable as the ventilatory activity of embryos creates cues that increase the risk of detection by predators. The embryos can reduce this risk by mediating their ventilation activity if they detect the presence of a predator using their acute electrosense. We tested the behavioural responses of three different age groups (1/3 developed, 2/3 developed and near hatching) of embryonic thornback rays *Raja clavata* to the presence of potential predators using predator-type electric field stimuli to determine how early in embryonic life predator elicited behavioural responses can occur. Egg capsules were exposed to continuous or intermittent stimuli in order to assess varying predator-type encounter scenarios on the ventilatory behaviour for the different developmental stages. All embryos reacted with a 'freeze response' following initial electric field exposure, ceasing ventilatory behaviour in response to predator presence, demonstrating innate functionality at the earliest possible stage in ontogeny. This ability coincides with the onset of egg ventilatory behaviour and may represent a sophisticated means to enhance survival. A continuous application of stimuli over time revealed that embryos can adapt their behaviour and resume normal activity, whereas when presented intermittently, the E-field resulted in a significant reduction in overall ventilatory activity across all ages. Recovery from stimuli was significantly quicker

in older embryos, indicative of the delicate trade-off between avoiding predation and adequate respiration.

Key words: Elasmobranch; electroreception; rajidae; predator avoidance; E-field

INTRODUCTION

Predator-avoidance responses rely on the individual sensory abilities of prey animals and are fundamental for survival. For life stages where physical escape from a predatory threat is not possible, it can be predicted that prey have evolved adaptations to reduce the chances of being detected. However, our understanding of the behavioural mechanisms that underpin such predator-prey interactions is impeded by a lack of studies to have directly assessed how anti-predator behaviours manifest and develop through the life history of an organism. One reason for this is that behavioural responses in the earliest life stages can be particularly difficult to study in many organisms, especially vertebrates. A small number of investigations considering oviparous vertebrates have demonstrated some significant effects of predator presence on hatching characteristics. Embryos of the salamander, *Ambystoma barbouri*, can delay hatching and consequently hatch at larger size in the presence of predators which feed on hatchlings but not on embryos (Sih and Moore, 1993; Moore et al. 1996). In contrast, embryos of the arboreal red-eyed tree frog (*Agalychnis callidryas*) have been shown to respond to the physical presence of a predatory snake by hatching immediately and falling into the water to increase likelihood of escape (Warkentin, 1995, 1999a). These studies clearly demonstrate that the mechanical and olfactory

sensory systems of embryos can function at this early stage to enhance survival. In addition, Oulton et al. (2013) recently demonstrated the innateness and adaptive nature of these kinds of responses in rainbow fish embryos; measuring an increased heart rate in response to the presence of a native predator as opposed to a novel one. Whilst being an obvious stress response which demonstrates the ability of embryos to detect predatory threats, this behavioural response does not present a means to avoid predation. Indeed very few studies have looked at the presence of predators and embryo behaviours *in situ* during the course of development. Hence, there remains a requirement for good model systems with which to address the questions that are central to our understanding of embryonic or early life behaviours which influence such predator-prey outcomes.

Oviparous elasmobranchs (sharks, skates and rays) represent an excellent system for studying predator-avoidance behaviour in early ontogeny. Their embryos spend the developmental phase encapsulated in an egg case which is attached to the seabed for periods of several months and its structure is sufficiently translucent for natural behaviours to be observed with little intrusion. Skates and rays (Family: Rajidae) deposit single embryo egg capsules that are characterised by four long horns extending from the capsule corners. Each horn terminates in a slit which is blocked in the early stages of development by vitelline jelly that surrounds the yolk mass (Luer and Gilbert, 1985). Around one-third into development, (the 'pre-hatch' stage), the jelly dissolves, allowing seawater to flow through the horn slits and into the capsule (Clark, 1922; Luer and Gilbert, 1985). Although the capsule membrane is permeable to various metabolites (Foulley and Mellinger, 1980; Evans, 1981), simple diffusion cannot sufficiently meet the corresponding metabolic demands of the growing embryo

(Long and Koob, 1997); hence at this stage, rajids begin to exhibit a rhythmic tail undulation in one of the four capsule horns, employing a specialist tail appendage which appears to be lost some time after hatching (Clark, 1922; Bigelow and Schroeder, 1953). This facilitates the necessary exchange of oxygen and waste metabolites, yet creates a localised vortex, providing olfactory or mechanosensory cues for predators, which may include fish and Gastropod molluscs (Cox and Koob, 1990; Lucifora and Garcia, 2004).

The tail beating has been shown to be interrupted in near hatching embryos of the clearnose skate (*Raja eglanteria*) by external phasic electric stimuli of 0.5-2 Hz, which matches the bioelectric fields produced by large fish predators during their own respiratory activity (Sisneros et al., 1998). This embryonic ‘freezing’ behaviour may momentarily remove sensory cues used by predators to detect skate embryos and potentially represents an important survival mechanism. Comparable responses have been reported in the catshark *Schyliorhinus canicula* (Peters and Evers, 1985) and banded bamboo shark *Chiloscyllium punctatum* (Kempster et al., 2013) who momentarily cease gill movements during late embryonic stages. However it remains to be demonstrated whether these sensory abilities extend to earlier embryonic stages. The pre-hatch stage signifies the start of ventilatory behaviour as a result of complete tail appendage development in skates which coincides with the disappearance of vitelline jelly (Hoff 2009). We hypothesised that this synchrony is further complemented by the presence of a sensory-mediated predator avoidance mechanism at this key stage in development, to enhance survival.

If the freeze response is a momentary reaction, presumed to improve chances of survival whilst a predator is in proximity of the egg capsule, it follows that, if not consumed, an embryo would need to behaviourally adapt to the electrical stimuli and resume ventilation in order to meet metabolic demands. Indeed adult elasmobranchs have been shown to habituate to electrical stimuli if they are presented with a consistent field strength from a constant source during foraging experiments (Kimber et al. 2013). It is likely that predator presence is variable through time; hence it is important to understand the effect of different exposures to predator type electrical stimuli on embryo behaviour which may shed light on the fundamental tradeoff between energy gain and predator avoidance. It could be predicted that a skate embryo experiencing an intermittent E-field would be more likely to exhibit repeat responses than if it were experiencing a constant field and the cost of that behaviour may vary depending on embryo age, yet this apparently has never been assessed. Long and Koob (1997) witnessed embryos of *R. erinacea* performing comparable ventilatory movements within the egg capsule from one third into development until hatching. They never quantified this behaviour, however Ganon (1991) recorded an exponential increase in oxygen consumption during embryonic development, which would correspond to the rapid increase in the rate of yolk sac absorption towards the end of development seen in *Bathyraja aleutica* and *B. parmifera* (Hoff 2009). With a higher metabolic demand, the need for tail beating is likely to increase, hence older embryos may be more conspicuous and prone to detection by predators. This increased vulnerability to predation and yet an increased requirement to ventilate with embryonic age may mean that the likelihood of freezing in response to electric field stimuli and habituation to such stimuli could differ between developmental stages.

Our knowledge of the development and consequences of anti-predator behaviour is hindered by a lack of amenable model systems. We quantified the ventilatory behaviour of embryos of the thornback ray, *Raja clavata* L. 1758 at different developmental stages to address fundamental knowledge gaps concerning the onset and patterns of predator-avoidance behaviour in the earliest relevant stages of ontogeny.

METHODS

Embryos of *R. clavata* were obtained from an established captive breeding programme at Blue Planet Aquarium, Cheshire, UK as freshly oviposited and tagged egg capsules. The breeding fish were originally caught off the west coast of Scotland. During this investigation, a total of 125 egg capsules were incubated in a large open circuit vessel (2m x 1m x 0.25m) supplied with natural seawater at the Port Erin Marine Laboratory, Isle of Man. Incubation temperature was maintained at 12°C in a constant temperature room.

A pilot study determined the total incubation period of these *R. clavata* embryos to be 27 weeks at 12°C. Three distinct age categories were identified as: 9-10 weeks (group A), 17-18 weeks (group B) and 26-27 weeks (group C) which represented embryos at one third into development (approximating the ‘pre-hatch’ stage), two thirds into development and near hatching respectively (following Long and Koob 1997).

Exposure to predator-type electric fields

Fifty-four embryos were randomly selected (representing 18 from each age group) and allocated either a control, constant or intermittent treatment. For the control, embryos experienced no E-field, for the constant treatment, they were exposed to a continuous E-field for a total of 40 minutes and for the intermittent treatment, the E-field was switched on or off at 10 minute intervals for the 40 minute duration.

During experimental trials, all egg capsules were individually suspended between carbon electrodes in a temperature controlled 18-l tank of natural seawater (Fig. 1) and allowed to acclimatise for 20 minutes. For the E-field treatments, a function generator was used to produce an alternating current (AC) electric stimulus at a frequency of 1-2Hz, thereby replicating a typical predator-type bioelectric field. Via an isolation amplifier and gold plated connectors, a sinusoidal uniform field was then applied to the carbon electrodes within the tank. A salt bridge electrode created an electric field intensity estimated as approximately $0.61\mu\text{Vcm}^{-1}$ (RMS) midway between the electrodes that was experienced by the embryos (Fig. 1). The field strength and frequency of treatments remained consistent throughout the study by regular monitoring of the applied voltage and current; only the pattern in which they were presented was altered.

A cold light source was used to backlight the activity of the embryos which was recorded by video camera. In synchrony with the E-field being switched on or off, a video marker was added onto the recording for later reference. Whether a 'freeze response' (cessation of tail movement when ventilating, or curling in of tail if stationary) occurred in each case was recorded from subsequent video analyses along with ventilatory activity which was quantified in each minute of the 40 minute exposure period as:

Tail Beat Frequency (TBF) - The number of tail beats per minute (where one beat was equal to one complete tail undulation, touching top and bottom of the entrance to egg capsule horn).

Time Spent Ventilating (VT) – Time spent ventilating an egg capsule as opposed to time spent resting per minute.

Over a two week period each individual was exposed to their assigned treatment on four occasions. The order in which the egg capsules were selected on a daily basis was randomised and minimum recovery period of 36 hours was factored in between each repeat treatment on a given individual.

Statistical analyses

Data exploration, statistical analyses and model validation (using AIC and residual-based methods) were carried out using R (R-Development-Core-Team). Statistical analyses determined: 1) the probability of occurrence of a freeze response; 2)

variation in recovery time following a freeze response; and 3) variation in ventilation time and tail beat frequency. To account for non-independence in the data arising from the four observations taken per individual, a mixed modelling approach was taken implementing the lme4 (Bates et al. 2014) and MASS (Venables and Ripley, 2002) packages in R, with individual embryo assigned as a random effect.

Response variables were modelled as functions of a set of covariates that corresponded to the questions outlined above. The probability of a response to an E-field was modelled using binomial errors, and as a function of embryo age (9-10 weeks; 17-18 weeks or 26-27 weeks), trial number (4 per individual), stimulus number (first or second for intermittent treatment), and whether the embryo was ventilating when the stimulus was applied.

The recovery time of an embryo following stimulation was modelled as the number of seconds using quasi-Poisson errors, and as a function of the same covariates used in the probability of a response analysis, in addition to a further variable describing whether an embryo showed a detectable response to the stimulus. Similarly, the tail beat frequency and ventilation time (number of tail beats, and seconds spent ventilating over the 40 minute trial period, respectively) were modelled using quasi-Poisson errors and the same set of covariates. Time was subsequently included as a categorical variable in order to contrast response levels at different time points for each of the treatments.

RESULTS

Probability of a Response to E-field stimulation

Embryos of each age category were seen to perform freeze responses. Unsurprisingly, the only factor that explained significant variation in the probability of a response to electric field stimulation was whether or not an embryo was ventilating at the time of stimulation ($Z = 4.35$; $P < 0.0001$; residual d.f. = 139), that is, there were relatively few visible responses if the embryo was stationary. Considering only the embryos that were ventilating at the time of onset of the electric field, only one individual (in the oldest age category) did not respond. Therefore, the overall probability of a response occurring was 97% regardless of other covariates. There was no significant variation in the probability of a response depending on embryonic age groups or repeated trial number. In addition, for embryos in the intermittent group, the occurrence of a response to the first stimulus did not predict the response to the second stimulus and both groups had a 48 % overall probability of responding.

Recovery time following stimulation

Significant variation in the time taken for an embryo to resume ventilation following an E-field stimulus was explained by trial number, and embryo age. Whilst controlling for trial number, on average, older embryos (group C) recovered 36 seconds more quickly than group B ($Z = -1.4$, $P = 0.17$) and 194 seconds more quickly than group A ($Z = -4.5$, $P = <0.0001$), with Group B recovering 158 seconds quicker than group A ($Z = -3.1$, $P = 0.002$; illustrated in Fig. 2). For the intermittent treatment, mean recovery time for the first stimulus was significantly shorter (by 151 seconds) than the second ($Z = 9.8$, $P = <0.0001$; note: these estimates are derived by holding other fixed effects constant, at trial number = 1, residual d.f. for the model = 24).

Ventilatory behaviour over time

There was a clear trend for reduced tail beat frequency (TBF) following changes in the electric field, particularly at the onset (Fig. 3). Contrasts between E field exposure type showed that, whilst controlling for other covariates, in minute 1 (representing the onset of the first E-field), both the constant and intermittent exposed individuals had significantly lower TBF than the control group ($Z = 4.44$ and 3.50 ; and $P = 0.0001$ and 0.001 respectively) but did not differ from each other. In minute 21 (representing the second onset of an E-field for the intermittent treatment only), TBF was significantly lower in the intermittent group than in both of the other groups ($Z = -4.84$; $P < 0.0001$). It was also lower in minutes 11 and 31 (representing the offset of E-field for intermittent treatment), compared with the constant treatment and control (illustrated in Fig. 3). These contrasts were significant for minute 31 (constant: $Z =$

2.64; $P = 0.013$ and control: $Z = 2.99$; $P = 0.005$) and marginally non-significant for minute 11 (constant: $Z = 2.52$; $P = 0.017$ and control: $Z = 1.92$; $P = 0.063$).

Overall ventilatory behaviour

Overall variation in the number of tail beats produced by an embryo over the 40 minute trial period was explained by treatment type and embryo age. Embryos exposed to an intermittent field had significantly fewer tail beats than those exposed to a constant field (d.f. = 31; $t = -2.26$; $P = 0.03$) or no electric field (d.f. = 31; $t = -2.70$; $P = 0.01$), though there was no significant difference between those two groups. All age groups differed significantly with tail beats of Group A < Group B < Group C (all contrasts d.f. = 31; $t > 3.26$; $P \leq 0.002$). Overall time dedicated to ventilation yielded a slightly different result. It was similarly explained by treatment type, embryo age and repeat trial number, with embryos exposed to an intermittent or constant electric field spent significantly less time ventilating than those not exposed ($t = -4.26$ and 2.45 ; and $P < 0.001$ and 0.029 respectively). However, there was no significant difference between those two groups ($Z = -1.85$; $P = 0.088$). Meaning overall tail beat number was the same, but following a freeze response, tail beating was faster.

Age group C (oldest embryos) spent a significantly higher proportion of time ventilating than age groups B ($t = 2.77$; $P = 0.016$) and A ($t = 3.48$; $P = 0.004$), with group A and B not differing significantly from each other, although the pattern was the same as for TBF (time spent ventilating by Group A < Group B < Group C).

DISCUSSION

The findings of this study provide novel insights into sensory-mediated predator-avoidance behaviour during ontogeny. We demonstrated that embryos of *R. clavata* exhibit adaptations consistent with avoiding predator detection at the earliest relevant stage of development; that this response decays with exposure and that the duration of recovery time following a response is age-dependent. In addition, across all developmental stages, embryos considerably alter their normal ventilatory behaviour when exposed to varying predator scenarios, potentially reflecting a fundamental trade-off between predator avoidance and respiratory requirements.

The anti-predator freeze response

The results clearly determined that embryos of each age category were able to detect E-fields as a sole stimulus. The primary sensory organ for the electrosense of elasmobranchs is the ampullae of Lorenzini. Freitas et al. (2006) demonstrated that the first recognizable stage of ampullary development in the cat shark *S. canicula* occurs as epidermal placodes around the pre-hatch stage (according to the staging system by Ballard et al., 1993). The ampullae, however, do not apparently form the

elongated tubes that connect to the surface of the skin until the point at which the yolk sac is practically empty and the fetus fills the egg capsule. The tubes are important for determining differences in electrical potential external to the fish when it is free swimming and enables E-field gradients to be detected and hence the direction of a stimulus to be determined (Tricas and Sisneros, 2004). During embryonic stages, the direction of a predatory stimulus is potentially not the most important factor, but simply the ability to detect and respond to a threat, thereby increasing the likelihood of survival. The results from our study suggest that the development of the ampullae alone at pre-hatch may provide sufficient means to be receptive to E-field stimuli, although histological analyses of skate embryos may reveal more. It is worth noting that certain differences exist between the embryology of skates and that of cat sharks, whose oviparity evolved along independent lines (Dulvy and Reynolds, 1997). Catshark egg capsules do not possess the long open horns of skate capsules and shark embryos do not have the specialized tail appendage (Leonard et al. 1999) which is undulated continuously until hatching, thereby making skates conspicuous to a variety of predators. The skate tail beating behaviour has been shown to be quite energetically demanding in near hatching *Raja erinacea*, yet considered necessary to meet associated metabolic demands that would not be satisfied by simple oxygen diffusion across the capsule (Leonard et al. 1999). It would seem fitting, therefore, that an efficient predator avoidance mechanism would be in place at the pre-hatch stage to support the requirement for this behaviour, providing an effective means to enhance survival. Skate egg capsules are known to be consumed by slow moving benthic gastropods and mobile fish. The latter have been demonstrated to emit low frequency AC electric fields of less than 3Hz, modulated by their own ventilatory movements (Bedore and Kajiura, 2013). The same has proven difficult to directly measure for

invertebrate molluscs such as Gastropods as they don't rhythmically pump water over gills (Bedore and Kajiura, 2013). However the ion leakage across mucous membranes is regarded as the greatest direct current (DC) source and results in a very low frequency AC-type field as a predator moves through the water. It is likely therefore that Gastropods also emit frequencies within the range coinciding with the peak frequency sensitivity previously demonstrated for near hatching embryonic skates (0.5-2Hz, Sisneros et al. 1998). Our results suggest that as early as one third into development, *R. clavata* embryos can detect and respond to the presence of predators that are likely to inhabit their coastal environment during incubation.

Owing to their trophic position, free-swimming elasmobranchs are seldom considered prey. However, defensive postures which might deter gape-limited predators have been recorded in young starry skates, *Amblyraja radiata* in response to being touched by divers (Martin and Rekdal 2006) and the southern thorny skate *Amblyraja doellojuradoi* when caught by trawlers (Cousseau et al., 2000). Cowtail stingrays *Pastinachus sephen* have been shown to preferentially rest with heterospecific whiprays, *Himantura uarnak* when visibility is poor in order to exploit their superior sensory perception, which improves the chances of predator detection and escape (Semeniuk and Dill 2004, 2006). Evidently, elasmobranchs may possess effective sensory-mediated predator avoidance mechanisms and strategies, yet their functionality and benefits to survival throughout ontogeny remain poorly understood.

Following an initial response, all skate embryos were seen to recover in the ongoing presence of an E-field and continued to ventilate their egg capsule. Recovery time was found to be shorter in older embryos, which may reflect a greater need to resume

ventilation owing to metabolic demands and could highlight a trade-off between fundamental respiratory and predator-avoidance behaviours. Gannon (1991) noted that the oxygen consumption rate for embryonic *R. erinacea* increased exponentially with body size, which has also been demonstrated in a number of larval teleosts (reviewed in Giguère Côté and St-Pierre, 1988). Concordant with this observation, the oldest embryos exhibited the highest overall levels of tail beat frequency and the youngest embryos, the lowest. Similarly older embryos dedicated significantly more time to ventilating than younger embryos, which is consistent with Thomason et al. (1996) who demonstrated that gill movement increased with body size during embryonic development in the catshark *S. canicula*.

Varying the predator scenario

Substantial differences were seen in the ventilatory patterns of all embryos when the presentation of the predator stimulus was altered over a 40 minute time period. During constant low frequency E-field exposure, tail ventilation resumed at a frequency comparable to a control, however, the 10 minute ‘field off’ period associated with the intermittent treatment proved sufficient for embryos to be re-stimulated, thus a secondary freeze response occurred when the E-field was switched on. Significant differences were apparent between recovery time length following first and second stimulations, with the second response being unexpectedly longer than the first. Obvious dips in tail beating following repeat stimuli meant that individuals experiencing the intermittent field recorded significantly fewer tail beats overall than the constant and control treatments. Reductions in tail beating at the onset of electric field stimulation and to some degree, the offset, highlighted the importance of

considering the effects of the time period when comparing treatments. Post and Von der Emde (1999) found that weakly electric fish *Gnathonemus petersii* altered their electric organ discharge rate in response to stimuli being switched off as well as on. Their explanation was that the onset of a stimulus evokes a novelty response because the sensory input deviates from the input of the recent past. However, when the stimulus remains on for some time, the fish builds up a new sensory template which includes the stimulus and therefore will additionally respond at its offset as it represents a novel environment. While this hasn't been studied in rajids, our results suggest that *R. clavata* embryos were adapting their behaviour following exposure. It has been demonstrated that free-swimming *R. erinacea* are capable of filtering out self-generated E-fields derived from the modulation of their own respiratory activity (Bodznick et al. 2002), however, whether elasmobranchs can employ an adaptive cancellation mechanism for expected extrinsic stimuli remains to be demonstrated. Certainly, sensitivity to novel stimuli can be considered evolutionary advantageous as it increases the likelihood of detecting imminent threats. Regardless of the underlying mechanism, it is remarkable that embryos as young as one third into development potentially exhibit such adaptations.

Embryos experiencing either an intermittent or a constant electric field spent significantly less time ventilating (which would make them less conspicuous to predators) than a control. The fact that those in the constant treatment group ventilated at approximately the same frequency overall to the control treatment suggests they did so at a higher rate. This potentially compensatory behaviour may demonstrate an ability to regulate oxygen uptake despite interruptions resulting from perceived predatory threats, however it would be necessary to additionally collect metabolic

data to fully demonstrate whether this is the case. Linear correlations have previously been documented between logarithmic oxygen consumption and tail beat frequencies in juvenile teleosts (Herskin and Steffensen 1998), hence it could be predicted that skate embryos ventilating their egg capsule at a lower rate may be drawing in less oxygen. Sufficient oxygen is considered crucial to the successful embryonic development of vertebrates and in skates simple diffusion across the egg capsule membrane cannot sufficiently meet the corresponding metabolic demands of growing embryos (Leonard et al. 1999; Richards et al. 1963).

Embryonic development represents a particularly significant life history stage, where for long periods immobile, encapsulated embryos may potentially be exposed to anthropogenic influence. Of particular concern is the significant increase in artificial electric fields associated with subsea electrical cables and networks across the coastal waters and seabed habitats (Gill et al., 2012). Further research should examine the effects of prolonged and varied electric field exposure (throughout an entire incubation period) on metabolism and subsequent hatchling success to assess whether any impacts on the recruitment rates of this and similar species would ensue. Like many elasmobranchs worldwide, *R. clavata* are slow growing, late-maturing fishes with a low fecundity; a set of life history traits which significantly increase their susceptibility to over fishing (Dulvy and Reynolds, 2002). Such vulnerability also means that studies concerning stressors on crucial life history aspects are needed if responsible management regimes are to be established.

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FIGURE LEGENDS

Figure 1. Experimental set up for electric field treatment.

Figure 2. Comparison of freeze response (recovery) time between embryonic age groups.

Figure 3. Tail beat frequency over time for different electric field treatments (green line = constant, blue line = intermittent, red line = control). Coloured dashed lines show 95% confidence intervals respectively. A clear dip in ventilatory activity can be seen at minutes 1 and 21 for the intermittent treatment, representing the onset of electric field stimuli.

Figure 1

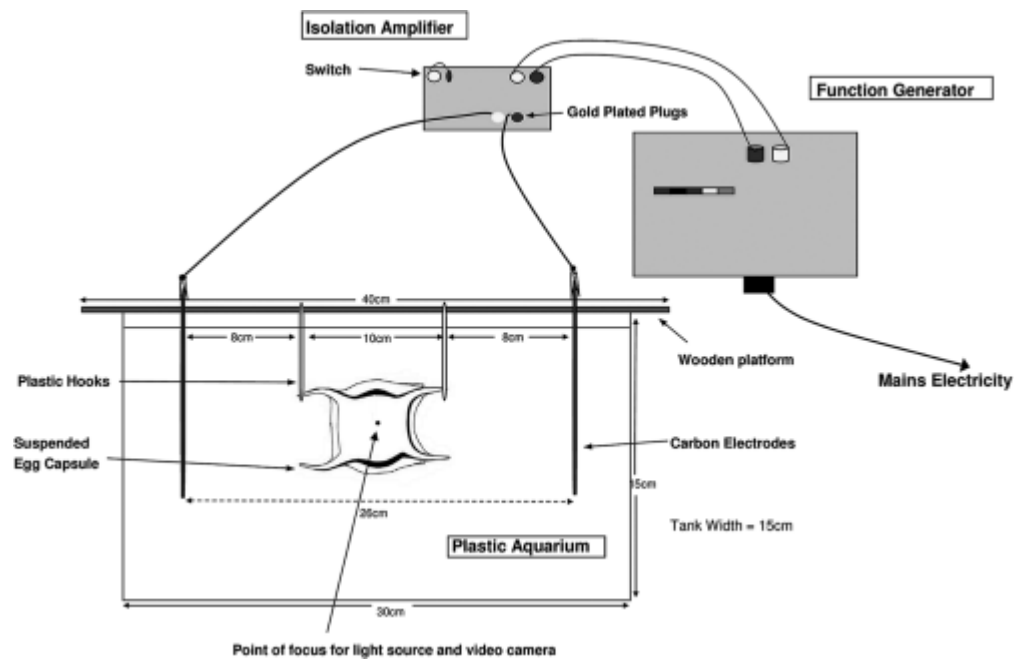


Figure 2.

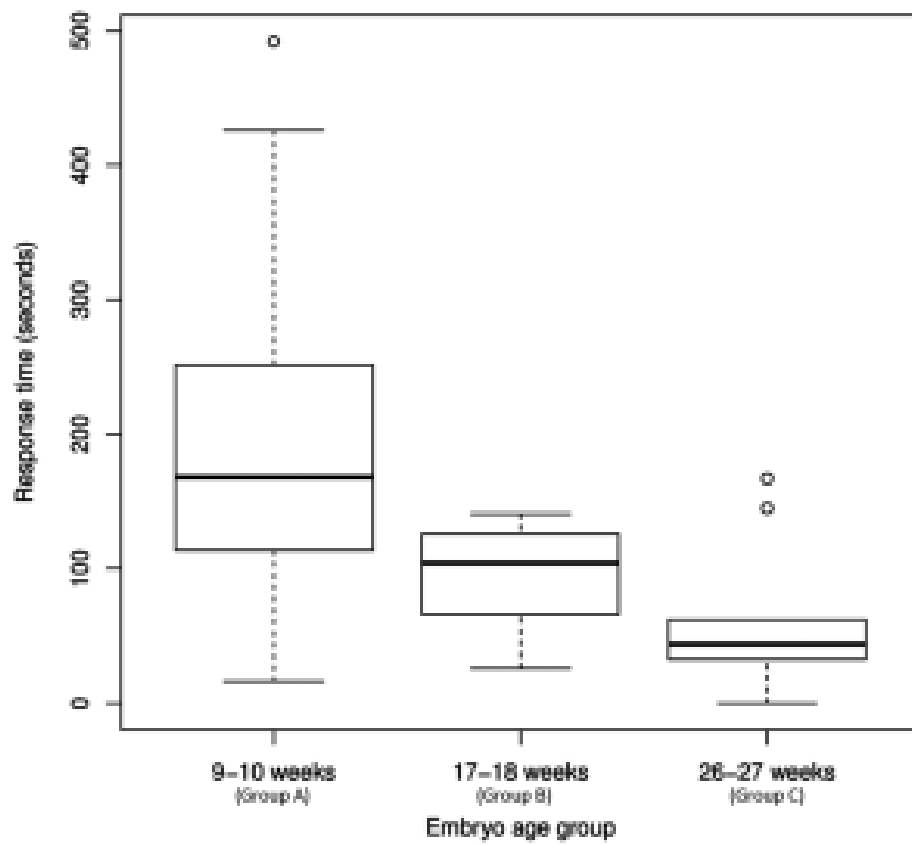
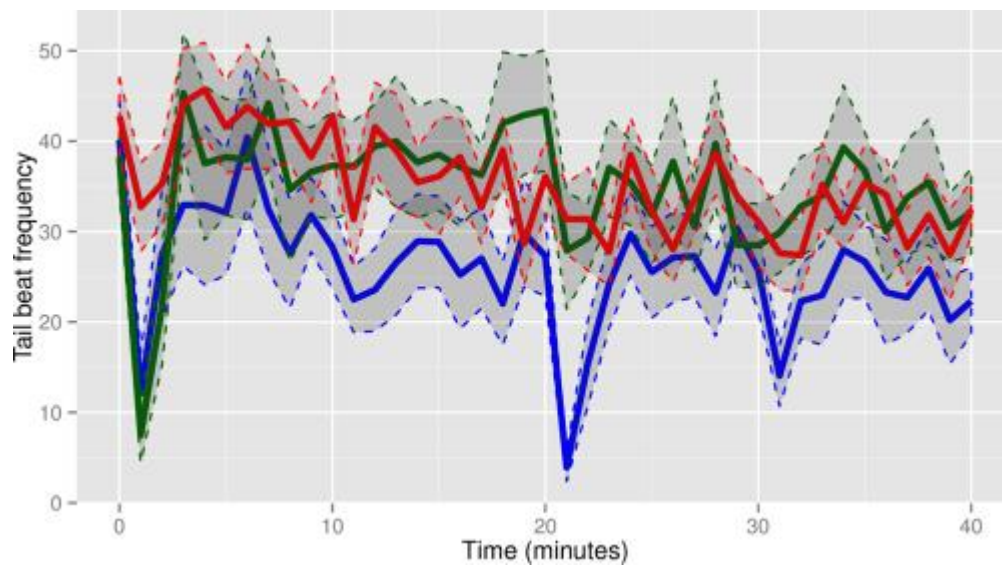


Figure 3





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Early life sensory ability—ventilatory responses of thornback ray embryos (*Raja clavata*) to predator-type electric fields

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