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Investigating the relative importance of parentage versus environmental factors on heterochrony in basommatophoran freshwater snails

Myriam Vanderzwalmen

Project Advisor: John Spicer, School of Marine Science and Engineering, Plymouth University, Drake Circus, Plymouth, UK, PL4 8AA

Abstract

The importance of genetics in inter-specific heterochrony is long known; however, intra-specific heterochrony was believed to be driven by environmental variation. Recent studies on *Radix balthica* showed higher similarity in developmental timing across generations in genetically similar embryos than in distantly related individuals. In the present study, the relative importance of parentage and egg-mass origin is compared with the environmental importance in generating the heterochrony in *Radix balthica*. Six developmental events were recorded daily. The analysis of the variations showed that parentage influencing variation in developmental timing to be more important than environmental conditions. Only hatching was affected by the environment as well as parentage. When comparing the sequence of developmental events, a strong link was found between treatments showing sequence variation and nil mortality in the same treatment. This was found between parents and between egg-masses from a single parent. This study shows that timing of developmental events is influenced by parentage whereas the sequence of developmental events is influenced by treatment. These findings indicate that both genetics and environmental plasticity influences intra-specific heterochrony but in different ways.

Keywords: heterochrony, environment, genetics, intra-specific variation, *Radix balthica*, evolution.

Introduction

An evolutionary link between ontogeny (an organism's development) and phylogeny (the evolution of taxonomical groups) was first proposed by Darwin [1] and DeBeer [2] who both believed that changes occurring during the development of organisms could become important in the evolution of new species and that it could be a mechanism driving speciation. Despite Darwin's [1] and DeBeer's [2] ideas of linking ontogeny and phylogeny, evolutionary studies tend to focus on either ontogeny or phylogeny but rarely on both. On the ontogeny level, studies tend to focus on differences observed between individuals of a single species or population. On the phylogeny level, species are studied to try to understand their evolutionary past and investigate what drives differences between the species. Heterochrony is an evolutionary concept linking ontogeny and phylogeny.

The interest in heterochrony, described as the variation in developmental timing between ancestor and descendant became prominent with Gould and his work 'Ontogeny and phylogeny' [3, 4]. Since the late 1970s the studies researching the link between ontogeny and phylogeny through heterochrony have mostly focused on comparing the development of species belonging to the same taxonomical group [e.g. 5, 6, 7]. In heterochrony existing integrated processes within organisms are shifted in time during an organism's ontogeny [8]. Heterochrony is a type of change during an organism's ontogeny on which selection can act and therefore drive evolutionary change.

Heterochrony is often studied by comparing the developmental timing and the sequence of developmental events of different species, ideally species closely related. Overall results show that heterochrony between different species is a result of genetic variation amongst the studied species [5, 9, 10] with some studies suggesting that the environment can produce phenotypic variation that can become genetically encoded heterochronic change over time [11, 12, 13]. The variation in the developmental timing within a single species however has long been believed to be a result of different environments acting on individuals and driving the observed changes [14].

A study by Smirthwaite *et al*. [9] on 12 basommatophoran snail species found extensive heterochrony between the species and showed that the observed heterochrony might have partially driven the evolution of the different species in the studied group. *Radix balthica*, one of the 12 species used by Smirthwaite *et al.* [9] has been found to show variation in the developmental timing between individuals within this species [15]. Recently Tills *et al*. [16, 17] suggested a potential genetic basis for timing differences between individuals of the snail *Radix balthica*. They found timing differences in the onset of developmental events between embryos of the same population. The genetic association became apparent when they found that individuals with the same time of onset of all developmental events studied had greater genetic similarities than individuals showing variation in the timings of development. This variation could provide the raw material on which selection may act on an intra-specific level leading to the formation of heterochrony on an inter-specific level hence linking ontogeny to phylogeny [15, 16, 17].

The aim of this study is to investigate the extent to which differences in timing of

developmental events and sequence of developmental events between individuals from the same population are generated by environmental stress as opposed to being determined by parentage or egg-mass origin between those individuals. This was investigated by exposing *Radix balthica* to increased salinity during the embryonic development and record the timing of six developmental events: the appearance of eye spots, the appearance of the heart, the timing of foot attachment, the beginning of crawling within the egg capsule, the beginning of radula movement and the timing of hatching [9]. The parentage and the egg-mass origin for each embryo were recorded to allow comparisons at the parental and egg-mass level. The effect of increased salinity on the physiology of developing embryos was investigated by measuring the daily heart rate of all the embryos. Comparing the development of the embryos sorted by treatment, parentage and egg-mass allowed the investigation of relative importance of each of those factors. We hypothesize that parentage will have a greater influence on the developmental timing of *Radix balthica* embryos as suggested by Tills *et al.* [16, 17]. The physiological response as well as the survival rate of embryos from the various parents could show possible increased adaptations to specific environments that are genetically based.

Climate change is accelerating environmental change through a variety of factors such as temperature increase; increase in CO_2 levels and for estuarine habitats, an increase in salinity cause by sea level rise levels. The ability of species to adapt to rapidly changing environments will affect their survival chances. These environmental changes will also influence ecologically driven evolutionary changes. It is therefore important to understand which mechanisms of evolutionary change the environment drives and which are driven by genetic variation.

The *Radix balthica* was chosen in this study, as it is an estuarine species known to have extensive variations in its developmental timing within the species that can be affected by salinity [15, 18]. *Radix balthica* was the species used by Tills *et al.* [19] to show a link between genetic differences and variation in the timing between individuals of the same population. In addition it is a species which breeds well in the laboratory and produces egg-masses frequently. It is a hermaphrodite species with asexual reproduction meaning offspring are genetically more closely related to the parents and siblings than sexually reproducing organisms would be. The embryos are small and transparent. It is therefore easy to observe the embryo's development under a microscope.

Materials and methods

Embryo collection and maintenance

Embryos used in the present study originated from a stock population collected from the River Dart in Totnes (Devon, UK 50.432° N, 3.684° W) at least a year ago. The population was maintained for three generations in a 15°C temperature control room. The individuals were exposed to natural daylight and maintained in artificial pond water (APW) (ASTP standard recipe: $MgSo^4$ 24.5; NaHCo3 19.5g; KCl 0.8g for 100L pH=7.4). The individuals were fed a 2 cm diameter piece of lettuce every three weeks. Each individual was kept in isolation (jar vol.= 268ml) and reproducing asexually, mostly lying egg-masses on *Elodea* pondweed. A total of 206 embryos were collected from 45 egg-masses originating from 23 different parents. From the

original 206 collected embryos from 23 different parents, the data from 90 embryos were retained originating from 4 parents. The parental origin as well as the egg-mass origin of each embryo was recorded in order to allow analysis of variation to be carried out knowing the parentage between each embryo.

Egg-masses were collected daily from the F3 generation. Embryos (with intact egg capsule) for use in the experiment were isolated from egg-masses that had not developed past the shell-ridge stage. Embryos were placed individually into wells of a 96 flat bottom multiwell plate (well capacity= 400 µl) (Sterilin, Ltd., UK) immediately after removal from the egg-mass. The embryos were exposed to either a control treatment of artificial pond water (see above) or artificial pond water with 3ppt of Instant Ocean™ salt (Na^{+}: 462 mmol kg^{-1}; K^{+}: 9.4 mmol kg^{-1}, Mg^{++}: 52 mmol kg^{-1}; Ca^{++}: 9.4 mmol kg^{-1}; Sr^{+}: 0.19 mmol kg^{-1}) [30] (pH= 7.4) throughout their development. The salinity level of the 3ppt treatment was checked daily using a hand refractometer (ATAGO, ChemLab Scientific Products Ltd., Japan) (S/MILL) and the pH of water from both treatments was measured using a pH-meter (Mettler Toledo FiveEasy) and adjusted, as necessary to maintain constant pH, using 0.1m HCl**.**

The embryos were placed randomly across the multiwell plate. A single embryo was placed per well, with five embryos of each treatment per plate. This was to avoid any possible “plate effect”. Water was changed daily to maintain constant salinity and pH levels as described above. The wells were filled to about ¾ to avoid desiccation of embryos due to evaporation. In addition, the multiwell plates were always covered with a lid to further prevent desiccation. Embryos were kept in a 20°C temperature control room with a 12h light/dark regime using 4 Aquaray™ Natural Day Light AO927 11-05-21 CXP-ND full spectrum at 20%.

Development recording

Following Smirthwaite *et al.* [9] seven morphological developmental stages known to show heterochrony in basommatophoran snails were recorded in the embryos: shell ridge, appearance of eye spots, appearance of the heart, attachment of the foot to the egg capsule/crawling, movement of radula and hatching (tab. 1 and fig. 1).

Observations were done on each embryo daily to record their development using an Optem™ x70 magnitude microscope. A daily photograph was taken of each of embryo using an ALLIED-PIKE F210C Vision Technologies™ imaging system mounted on the microscope and an AVT Smartview ™1.11 software system. A photograph of a measuring scale (GRATICULES Ltd. Tonbridge, Kent, England UK) (scale: 100x0.1= 10mm) was also taken with each used magnification to allow standardised measurements of embryo length and width. These measurements were subsequently used to calculate egg volume. The volume was calculated for each day of development for all embryos following Taylor [20]. The used equation was: $1/6\pi LW^2$ (L: length W: width).

Physiological changes were tracked daily by heart beats per minute starting the day the heart appeared or once the heart rate was regular enough to allow measurement to be taken.

Once hatched, embryos were transferred into glass jars (vol.= 35ml) containing water of the treatment in which they developed, a five cm long piece of Canadian pondweed, *Elodea* was added to the jar. Hatched embryos were maintained in the same 20°C temperature control room with 12h light/dark regiment as the one used

for embryo housing (see above). Four weeks after hatching, an eight mm in diameter piece of lettuce was fed to the hatchlings.

Event	Description
Shell ridge formation	Start of distinction between shell and developing foot
Eyes spot appearance	Formation of eye spots with darkened pigmentation
Heart appearance	Movement of heart chambers
Foot attachment	Foot attaches to egg capsule
Crawling	Crawling within the egg capsule
Radula movement	Forward motion of radula appendix within the head area
Hatching	Rupture of egg capsule and subsequent free crawling of juvenile

Table 1: Descriptions of the developmental events and stages recorded [9]. Each developmental stage and event was recorded when it was first observed, observations were made every 24 h

Statistical analysis

The timing of developmental events was recorded from the time the shell ridge appeared and not collection time. This was done due to the fact that collected embryos were at various stages of gastrulation and the start of development had to be a standardized and an identifiable event for correct analysis. Two-way nested ANOVAs were carried out for each developmental event and daily heart rates using Minitab 16 Statistical Software. The ANOVAs were carried out using the general linear model. Parental origin and water treatment were set variables and egg-mass origin and egg volume was treated as a co-variable. Egg-mass origin and egg volume were set as co-variables to detect any possible effect on the developmental timing below the level of parental origin.

The data were filtered after initial statistical analysis due to high levels of error linked to a high amount of embryos originating from parents which produced a small number of embryos (i.e.: average of only one, two or three embryos). Reducing the overall number of embryos increases the robustness of the statistical analysis carried out. Out of the initial 23 parents and the 206 hatched embryos, four parents were selected to have produced enough offspring to be analysed and the data from a total of 90 embryos remained. The same ANOVAs with the same factors and co-variables settings were carried out of the filtered data.

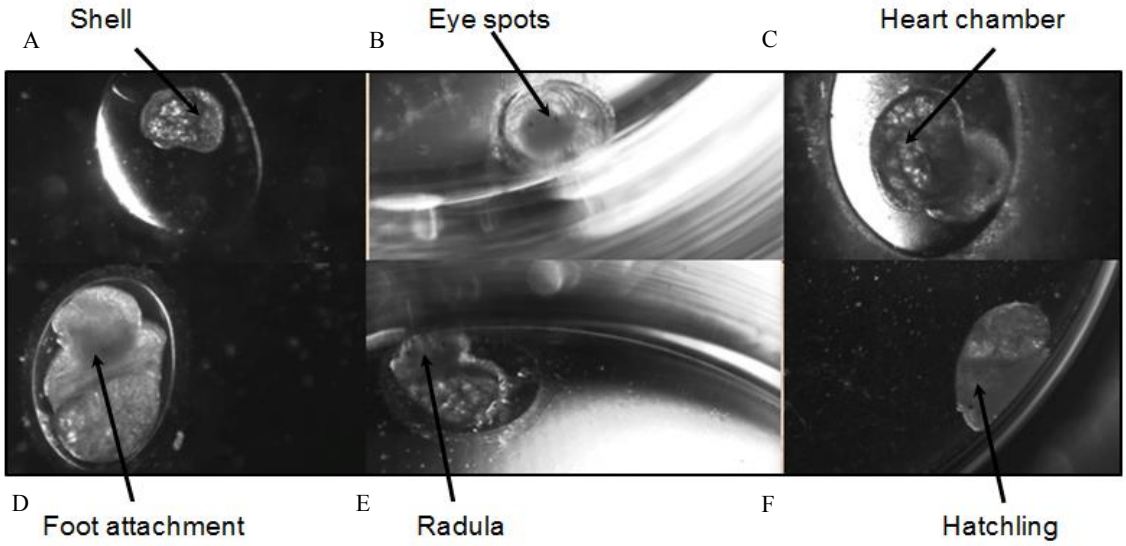

Figure 1: Developmental stages of *Radix balthica* [9]: A) appearance of shell ridge B) appearance of eye spots C) appearance of heart chambers D) attachment of the foot onto egg capsule E) beginning of radula movement F) hatched embryo.

The variation in the sequence of developmental events was compared by plotting on a pantograph the order and the timing of developmental events by parentage and by treatment. Each observed sequence variation for each parent was plotted against the most general developmental sequence from the respective parent. This allowed visual comparison of any variation in the sequence of developmental events. Each egg-mass showing variation for parent 1 was plotted on a pantograph as well to allow comparisons of the developmental sequence between the egg-masses and treatment to which the embryos were exposed. Again, each sequence variation was plotted against the most common sequence from the respective egg-mass.

Mortality was assessed for the embryos collected from the four selected parents. The mortality was calculated in percentage for the total number of embryos produced by each parent. Mortality had to be calculated this way because each parent produced a different number of embryos therefore calculating the percentage allowed standardisation between the parents. Mortality per treatment was assessed for the egg-masses from parent 1 to allow analysing the effect of increased salinity below the level of parent.

Results

Alterations in the timing of developmental events in embryos that survived

Developmental time of events showed less variation between embryos that originated from the same parents than between those that originated from different parents. However within a parent, the timing of developmental events was different for the two treatments. Increased salinity tended to delay the development to varying degrees between the different events as well as to a varying degree between the parents [Fig. 2-7]. The majority of events were slowed down by increased salinity, with the exception of the timing of heart appearance in parent 1 [fig. 3] and the timing

of radula movement in parent 2 [fig. 6] where the timing of development in the control occurred later than in the increased salinity. This variation however was minimal compared to the other variations. A Two-way ANOVA using General Linear Model was carried out on each developmental event separately to test for the relative effect of parental origin, egg-mass origin and exposed treatment. All four factors, parental origin, egg-mass origin, treatment and egg volume had no significant effect on developmental timing of eye spots (n= 76 F= 0.15 p= 0.927, F= 0.01 p= 0.926 and F= 0.89 p= 0.350, F= 0.07 p= 0.791 respectively R^2= 2.73%). The timing of the appearance of the heart was dependent on the parental and egg-mass origins (n= 76 F=2.90 p= 0.042 and F= 8.34 p = 0.005 respectively R^2= 19.44%). The treatment and egg volume had no significant effect on the timing of heart appearance (n= 76 F= 0.53 p= 0.470, F= 1.74 p= 0.191 respectively). The timing of foot attachment and the start of crawling were observed simultaneously except for one instance and the results of the statistical analyses are therefore reported together. The parental origin was found to have a significant effect on the timing of foot attachment and crawling (n= 76 F= 3.80 and F= 3.88 respectively $p < 0.02$ for both events R^2= 18.89%). However the egg-mass origin, the treatment and the egg volume had no significant effect on the timing of foot attachment and crawling (n= 76 F= 0.51 $p > 0.4$ and F= 1.85 $p > 0.1$, F= 0.51 $p> 0.4$ respectively). The timing of radula movement was significantly varying when analysing the parental origin (n= 76 F= 2.75 p = 0.05 R^2= 14.81%) however egg-mass origin, the treatment and egg volume had no significant effect on developmental timing (n= 76 F= 1.45 $p > 0.2$ and F= 0.40 $p > 0.5$, F= 0.45 p= 0.504 respectively). The timing of hatching was the event found to have the most variation. Both parental origin and treatment were found to significantly affect the timing of hatching (n= 76 F= 6.90 $p > 0.001$ and F= 6.29 $p > 0.01$ respectively R^2= 35.14%). The egg-mass and the egg volume had no significant effect on the timing of hatching (n=76 F= 0.11 p =0.739 and F= 1.37 p = 0.246 respectively).

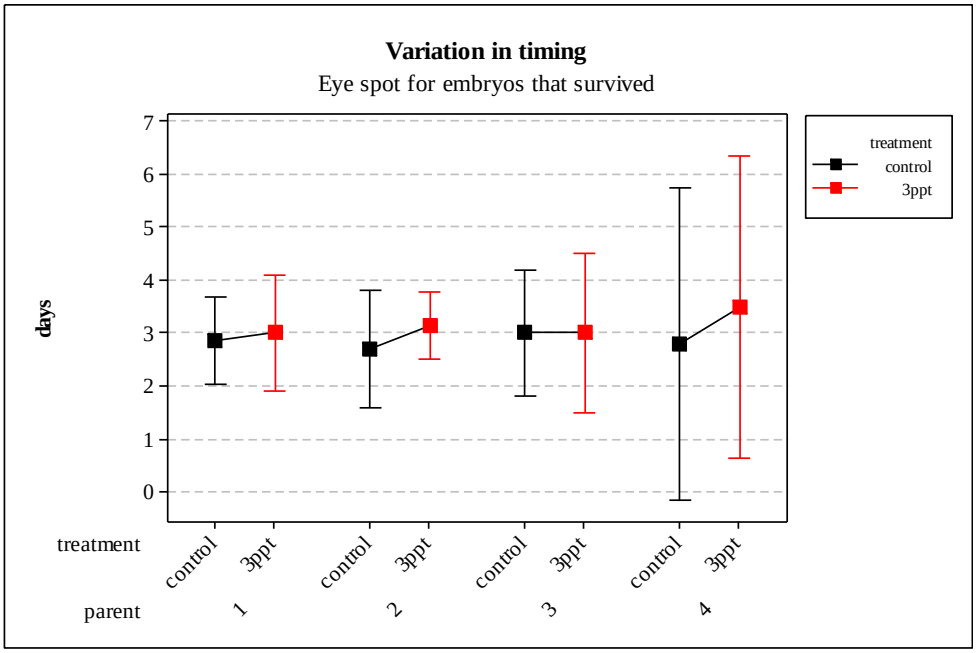

Figure 2: Mean (±95% CI) variation in timing of eye spot appearance in *R. balthica* by parentage and treatment (control and 3ppt) for embryos that survived post hatching

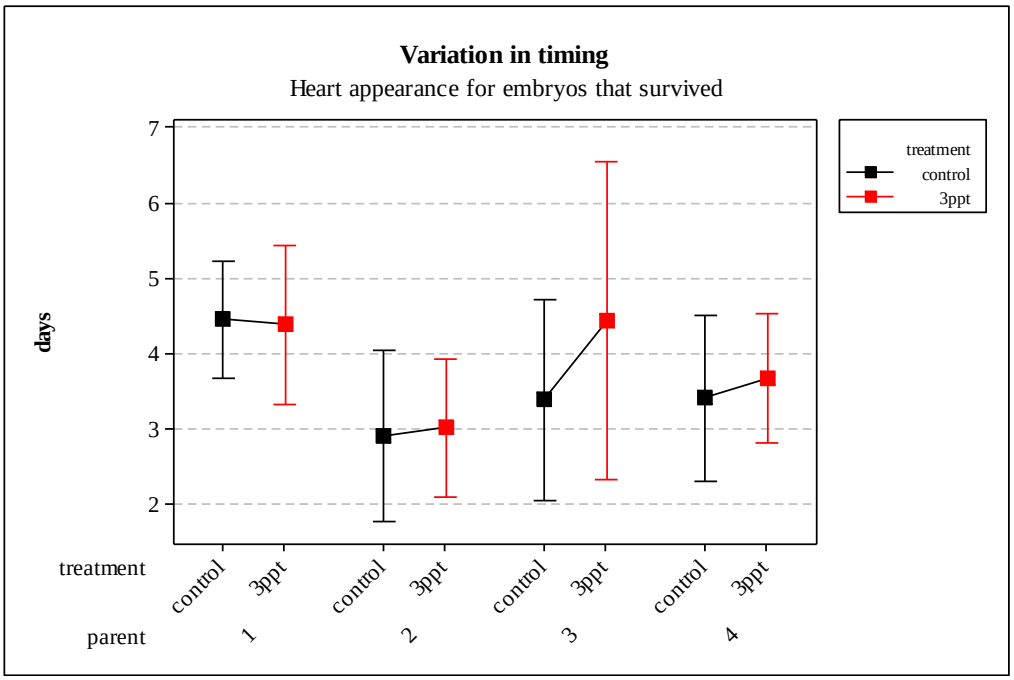

Figure 3: Mean (±95% CI) variation in timing of heart appearance in *R. balthica* by parentage and treatment (control and 3ppt) for embryos that survived post hatching

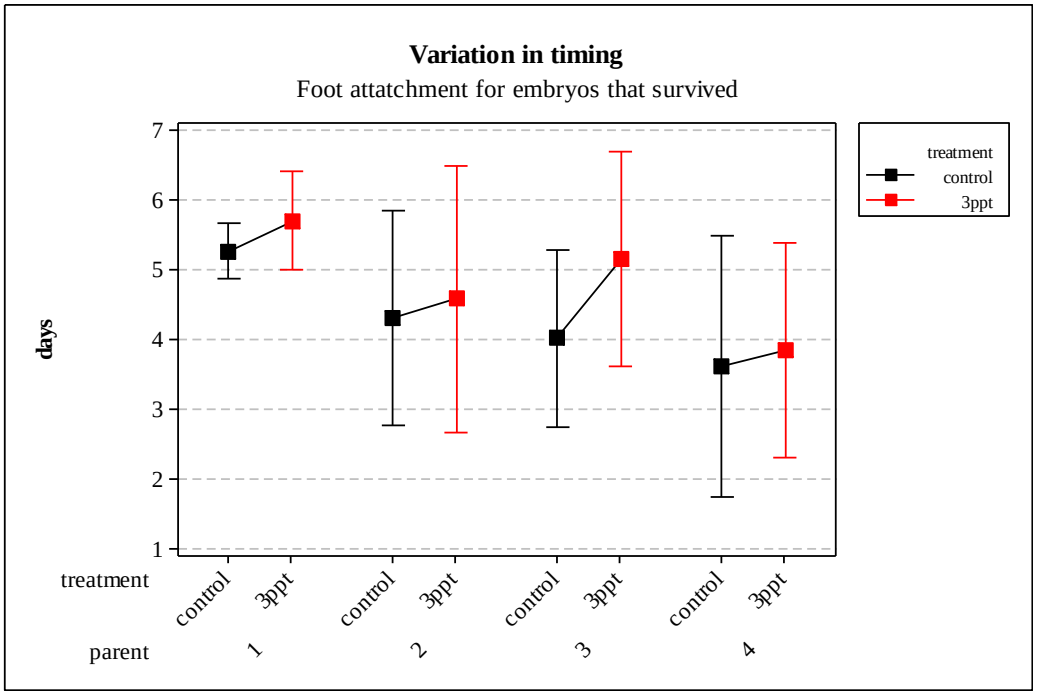

Figure 4: Mean (±95% CI) variation in timing of foot attachment in *R. balthica* by parentage and treatment (control and 3ppt) for embryos that survived post hatching

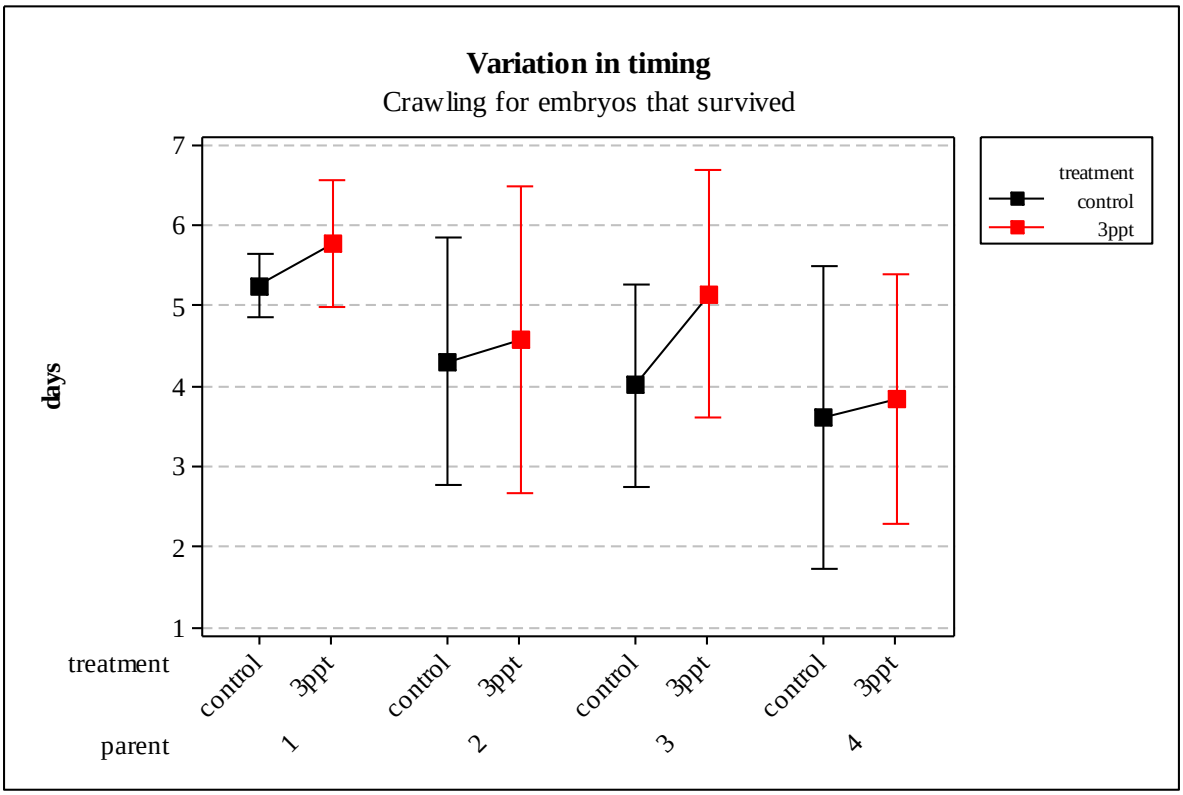

Figure 5: Mean (±95% CI) variation in timing of crawling in *R. balthica* by parentage and treatment (control and 3ppt) for embryos that survived post hatching

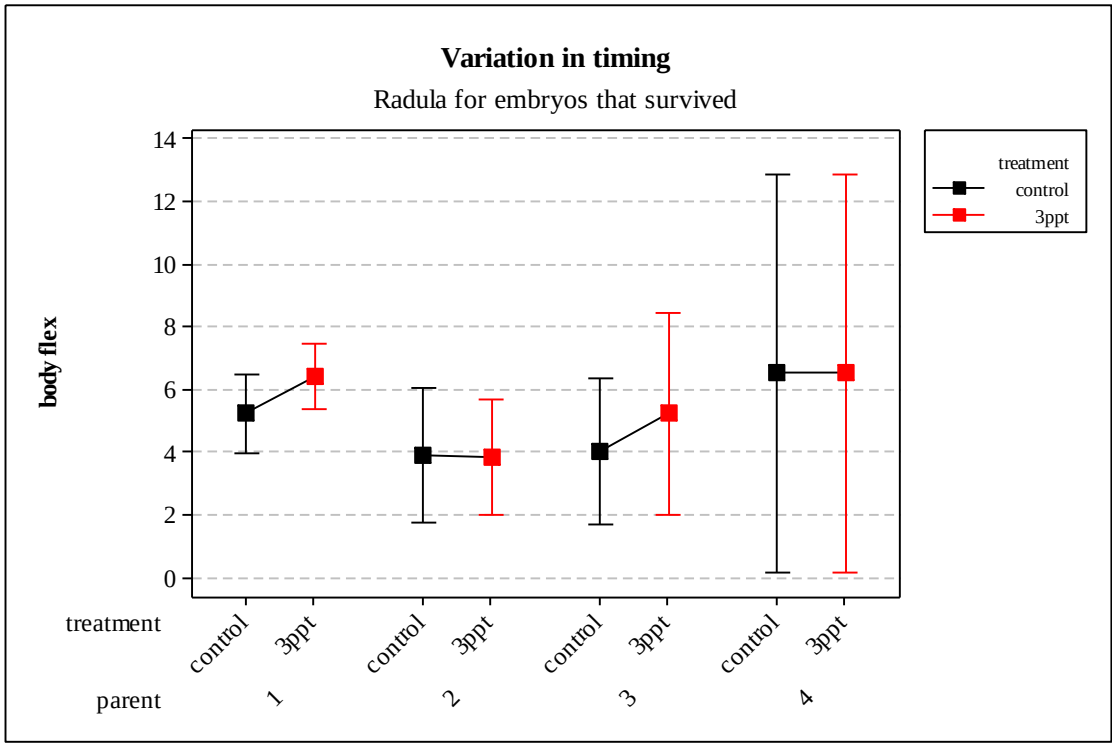

Figure 6: Mean (±95% CI) variation in timing of radula movement in *R. balthica* by parentage and treatment (control and 3ppt) for embryos that survived post hatching

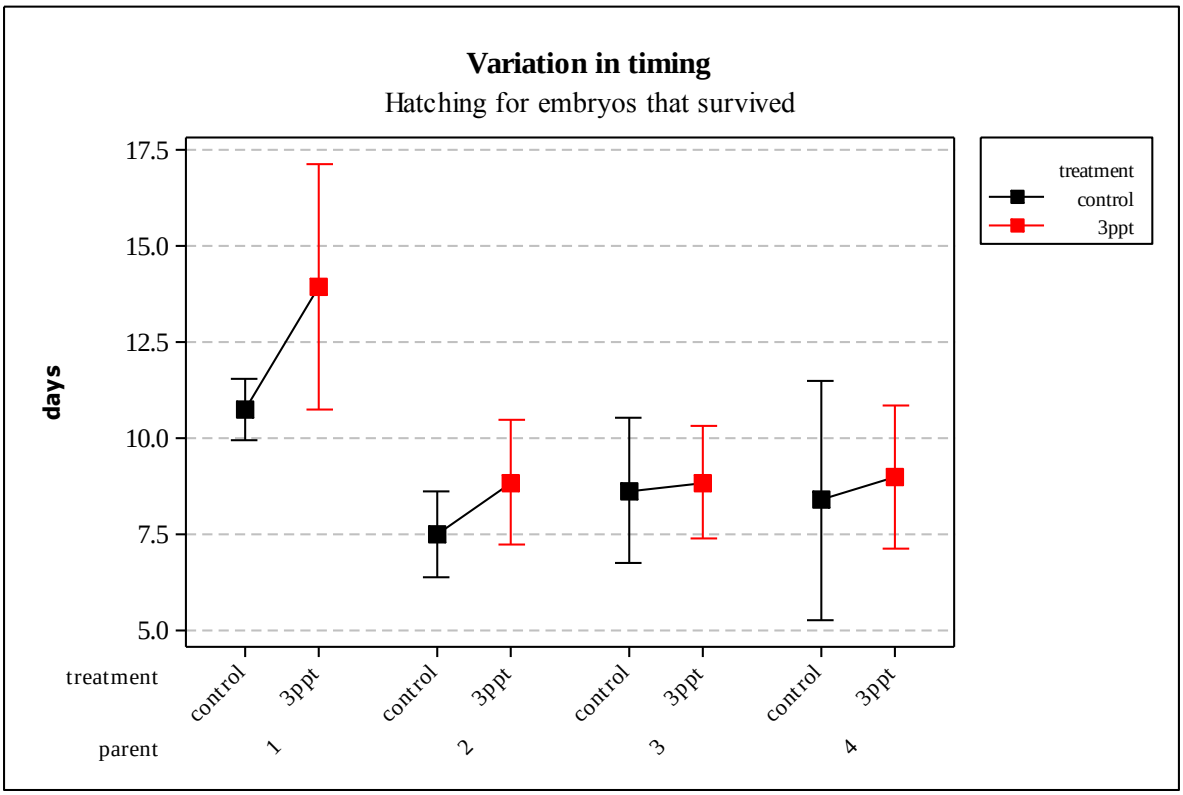

Figure 7: Mean (±95% CI) variation in timing of hatching in *R. balthica* by parentage and treatment (control and 3ppt) for embryos that survived post hatching

Alterations in the timing of developmental events in embryos that died

The same analyses were carried out for the embryos that died during their development. Analysing embryos that died during their development did not show a clear trend in the developmental timing similar to the one found for embryos that survived. Instead increased salinity seemed to both slow down and accelerate the timing of certain events with no clear pattern found [Fig. 8-12]. For all of the events, the only significant effects obtained were by the egg-mass origin on the timing of foot attachment and crawling (n= 16 *F*= 2.00 and *F*= 18.00 respectively $p < 0.02$ for both events) which were the events that were significantly influenced by egg-mass and parental origin and are the two effects that were found to have a genetic basis as found by Tills *et al.* [16]. Parental origin was also found to have a significant effect on the timing of foot attachment and crawling in embryos (n= 16 *F*= 55.37 and *F*= 56.47 respectively $p < 0.02$ for both events).

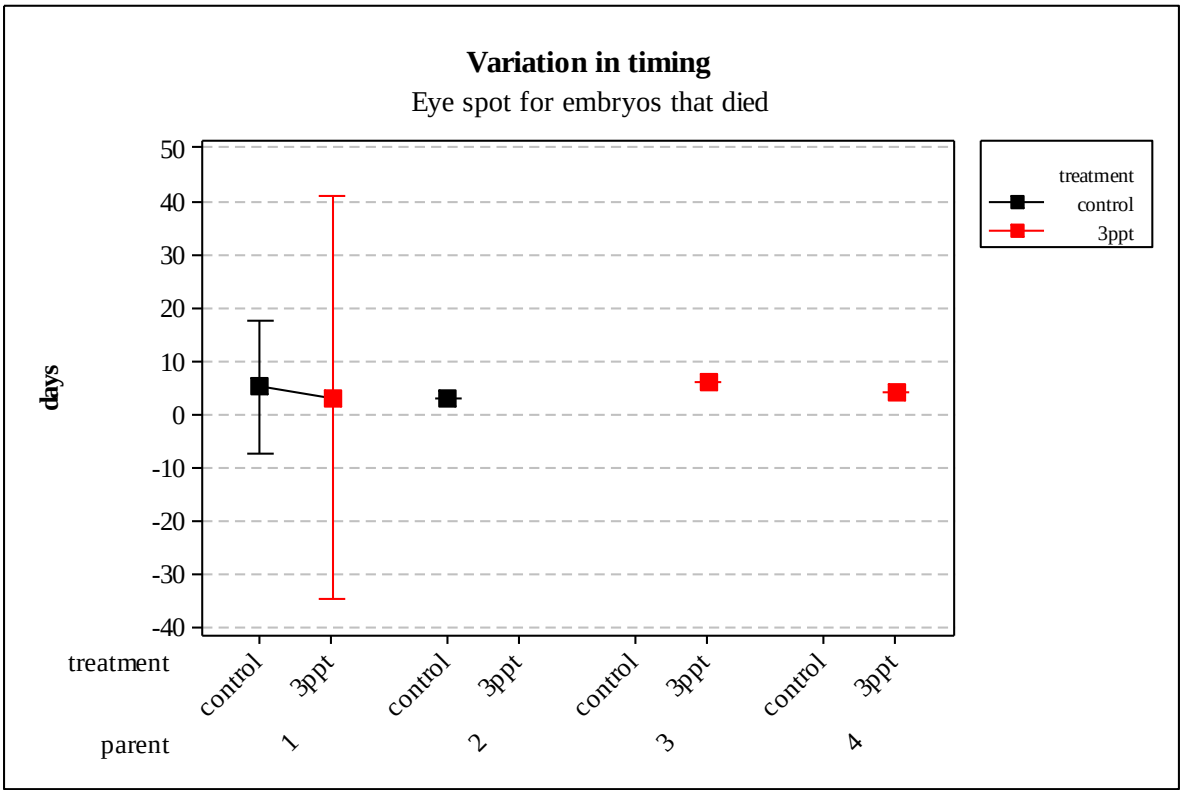

Figure 8: Mean (±95% CI) variation in timing of eye spot appearance in *R. balthica* by parentage and treatment (control and 3ppt) for embryos that died during development

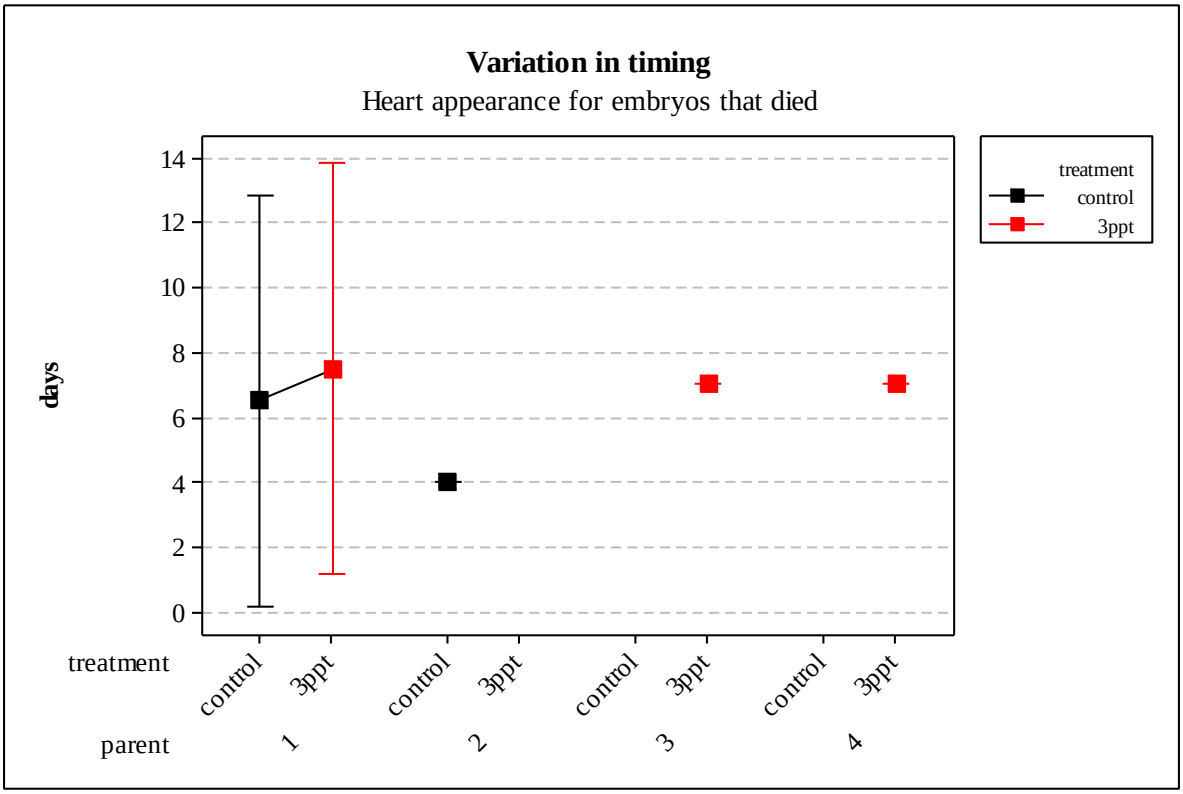

Figure 9: Mean (±95% CI) variation in timing of heart appearance in *R. balthica* by parentage and treatment (control and 3ppt) for embryos that died during development

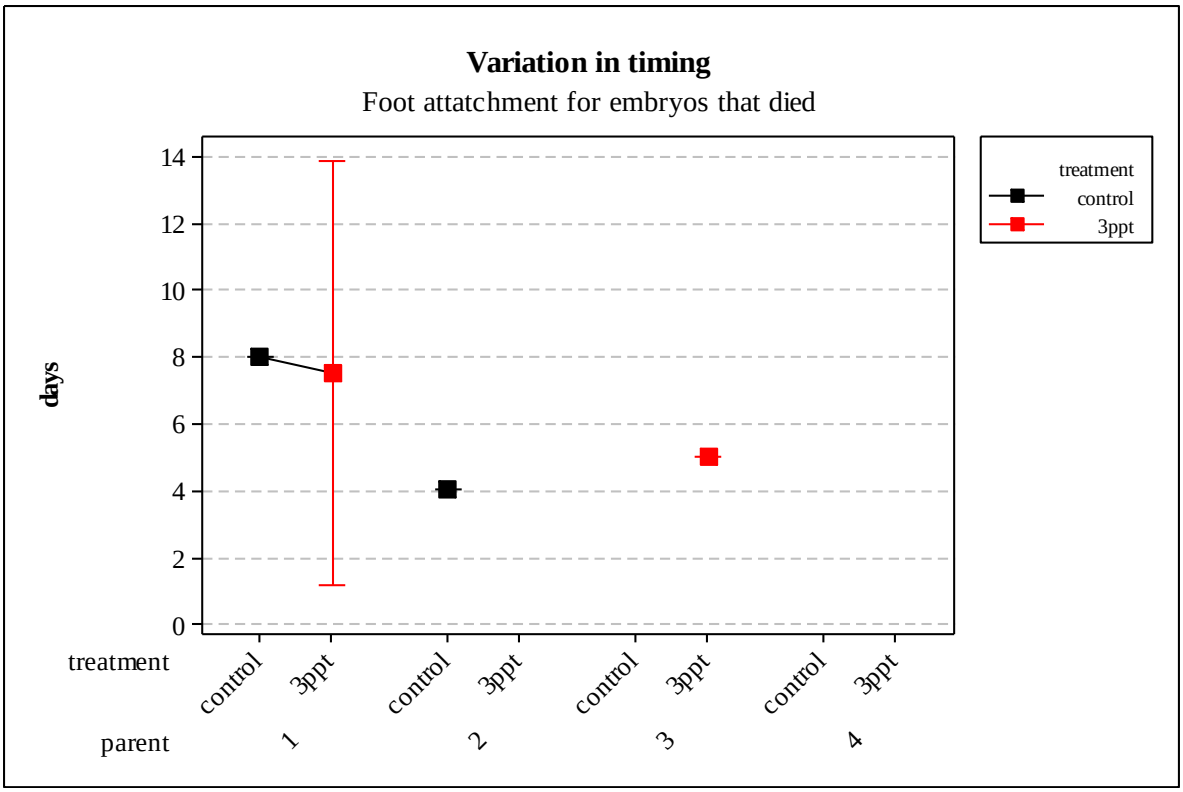

Figure 10: Mean (±95% CI) variation in timing of foot attachment in *R. balthica* by parentage and treatment (control and 3ppt) for embryos that died during development

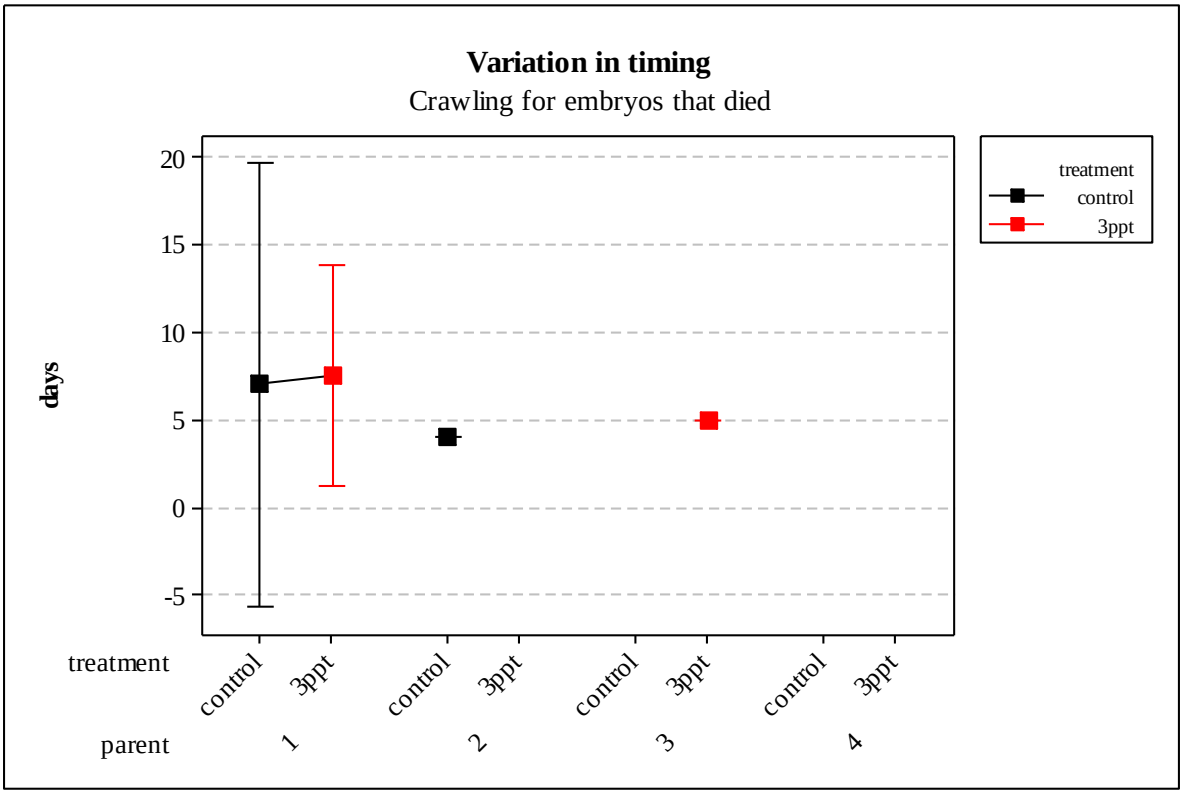

Figure 11: Mean (±95% CI) variation in timing of crawling in *R. balthica* by parentage and treatment (control and 3ppt) for embryos that died during development

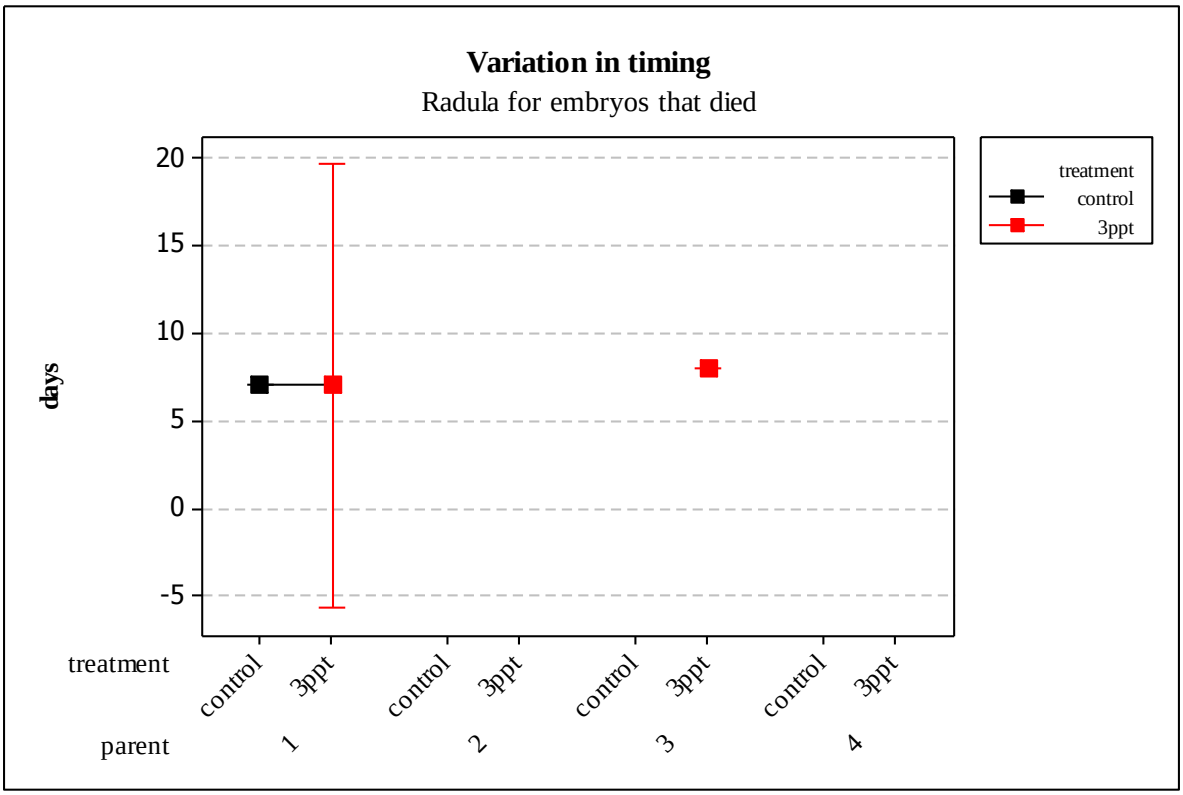

Figure 12: Mean (±95% CI) variation in timing of radula movement in *R. balthica* by parentage and treatment (control and 3ppt) for embryos that died during development

Alteration in the sequence of developmental timing

The comparisons of the sequence of developmental events plotted on a pantograph showed that the embryos of parent 1 and parent 4 had undergone variation in the sequence of development in both the control APW treatment and the 3ppt salinity APW treatment. The embryos of parent 2 and parent 3 however, showed variation in sequence of development in only one treatment (3ppt salinity APW for parent 2 and control APW for parent 3) [Fig. 13]. There was no consistency found in the variation of the order of developmental events within either treatment.

The comparisons of the sequence of developmental events within a parent that showed variation in both the control APW and the 3ppt salinity APW, was done by comparing different egg-masses from parent 1. Embryos in five egg-masses out of nine from parent 1 showed variation in the sequence of developmental events. Three egg-masses showed variation only in the control APW and two showed variation in both the control APW and the 3ppt salinity APW. There was no consistency found in the variation of the order within either treatment, similar to what was found when comparing the sequence between parents.

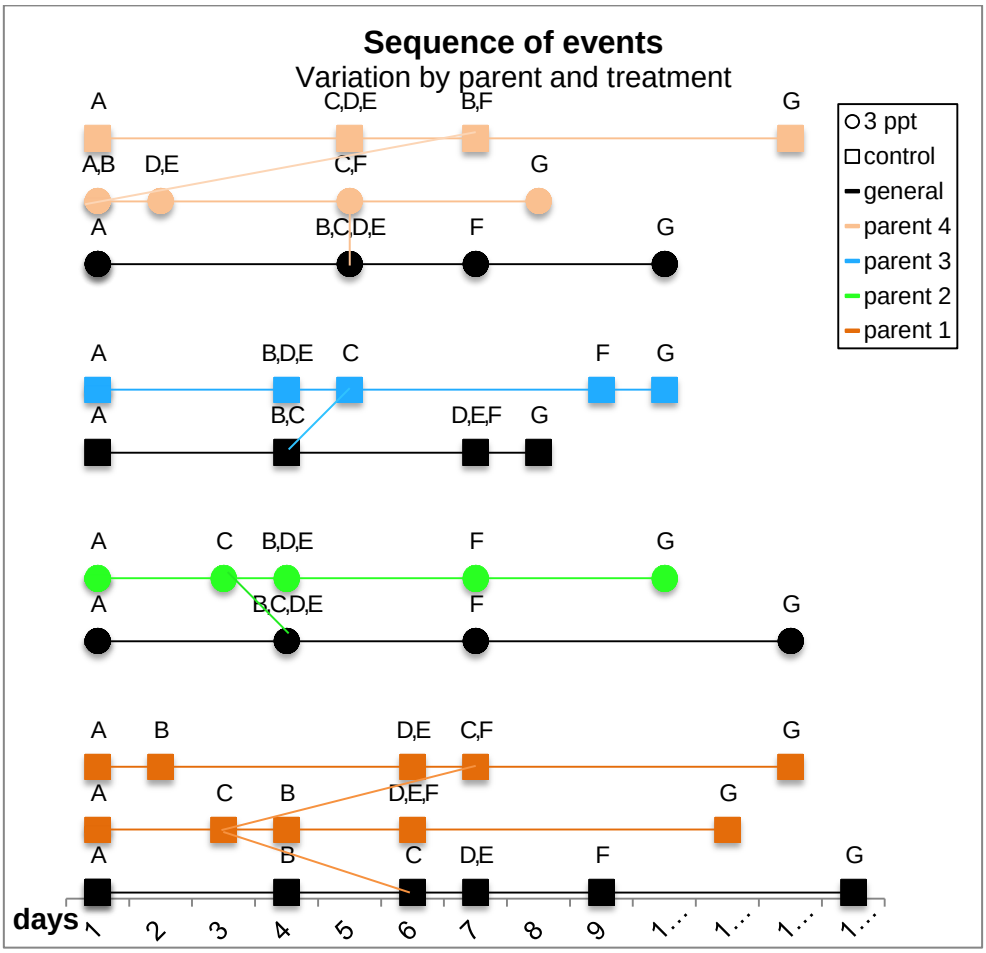

Figure 13: Changes in the sequence of developmental events *R. balthica* by parent and treatment. A) appearance of shell ridge B) appearance of eye spots C) appearance of heart chambers D) attachment of the foot onto egg capsule E) beginning of radula movement F) movement of radula G) hatched embryo

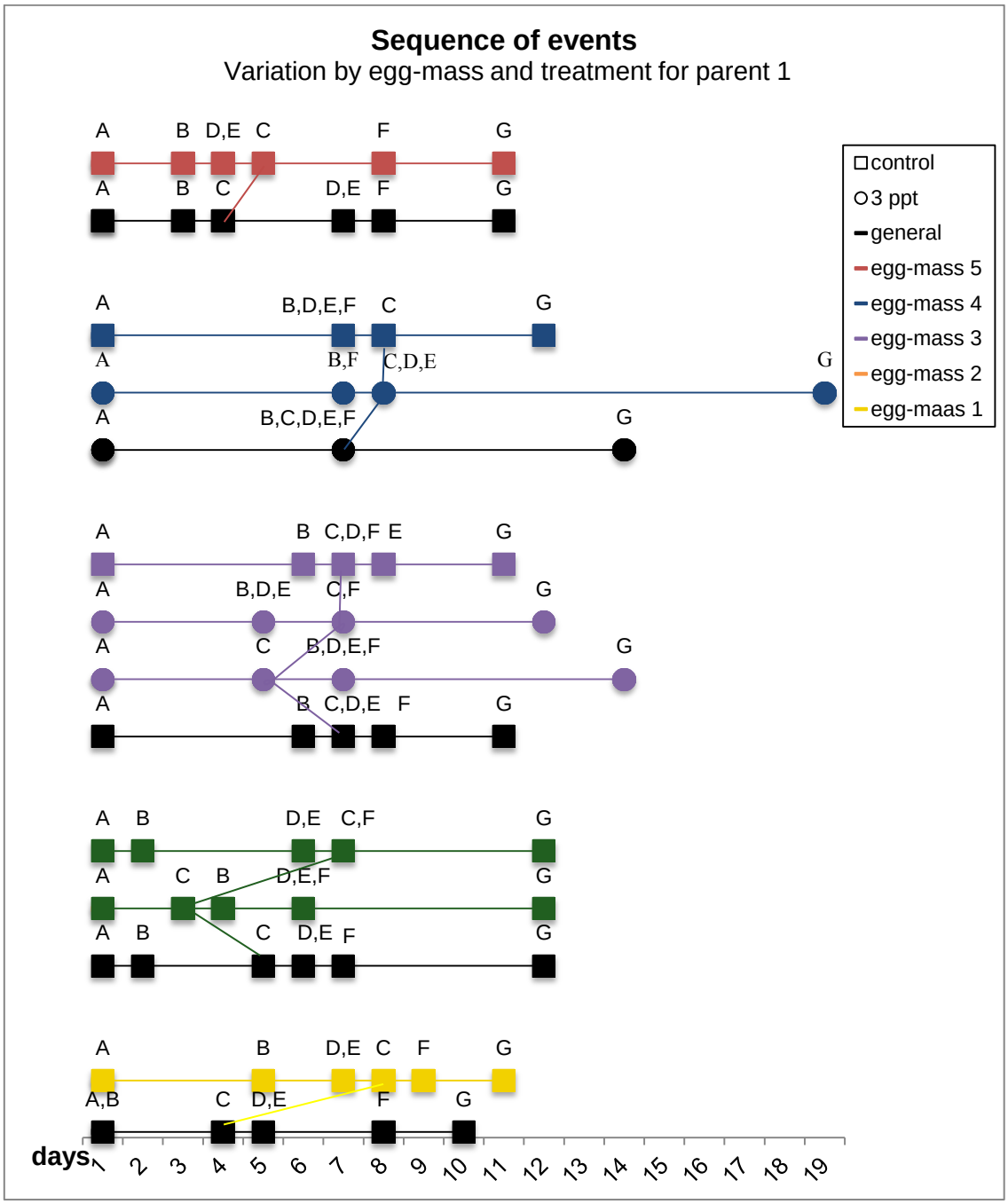

Figure 14: Changes in the sequence of developmental events *R. balthica* by egg-mass and treatment for parent 1. A) appearance of shell ridge B) appearance of eye spots C) appearance of heart chambers D) attachment of the foot onto egg capsule E) beginning of radula movement F) movement of radula G) hatched embryo

Heart rate

Daily heart rates for the selected parents were analysed using two-way nested ANOVA using General Linear Model. Neither the treatment nor the parental origin/egg-mass was found to have a significant influence on the daily heart rate of the embryos [fig. 15 and 16].

Mortality

From the original 206 collected embryos from 23 different parents, 106 embryos survived and 26 died before they hatched. 74 embryos were lost during regular maintenance; the data from 90 embryos were retained originating from 4 parents. Of those 90 embryos, 76 embryos hatched and 14 died during their development. Out of the remaining 14 embryos from the filtered data, 8 embryos died for the control APW treatment and 6 died for the 3ppt APW treatment. The mortality data was assessed for each treatment and parent of the filtered data. Parent 1 and parent 4 had a mortality rate between 15-35% in both treatments. Parents 2 and 3 however had mortality in only one treatment with nil mortality in the other treatment [fig. 17]. Mortality per treatment was assessed for the egg-masses from parent 1. All egg-masses showed mortality in the 3ppt salinity APW treatment except for the egg-masses with nil mortality. Three egg-masses showed mortality in the control APW as well as in the 3ppt salinity APW [fig. 18].

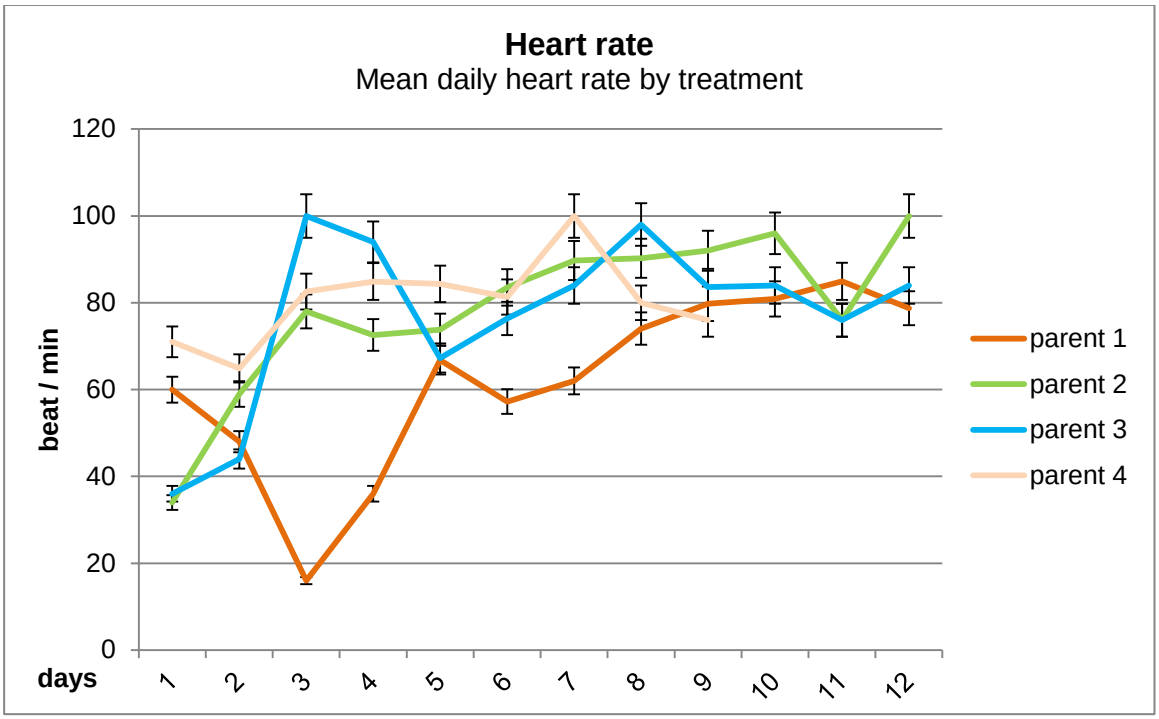

Figure 15: Mean daily (±95% CI) heart rate (beat/min) for embryos that survived by parental origin

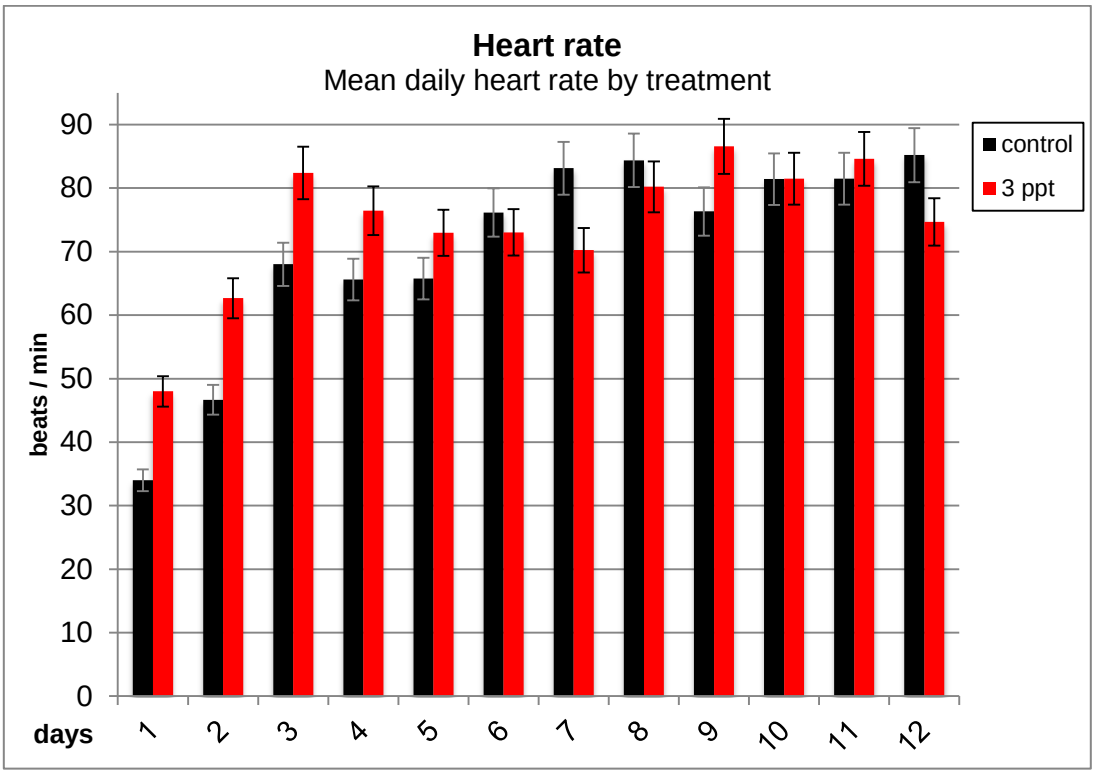

Figure 16: Mean (±95% CI) daily heart rate (beat/min) for embryos that survived by treatment (control and 3ppt)

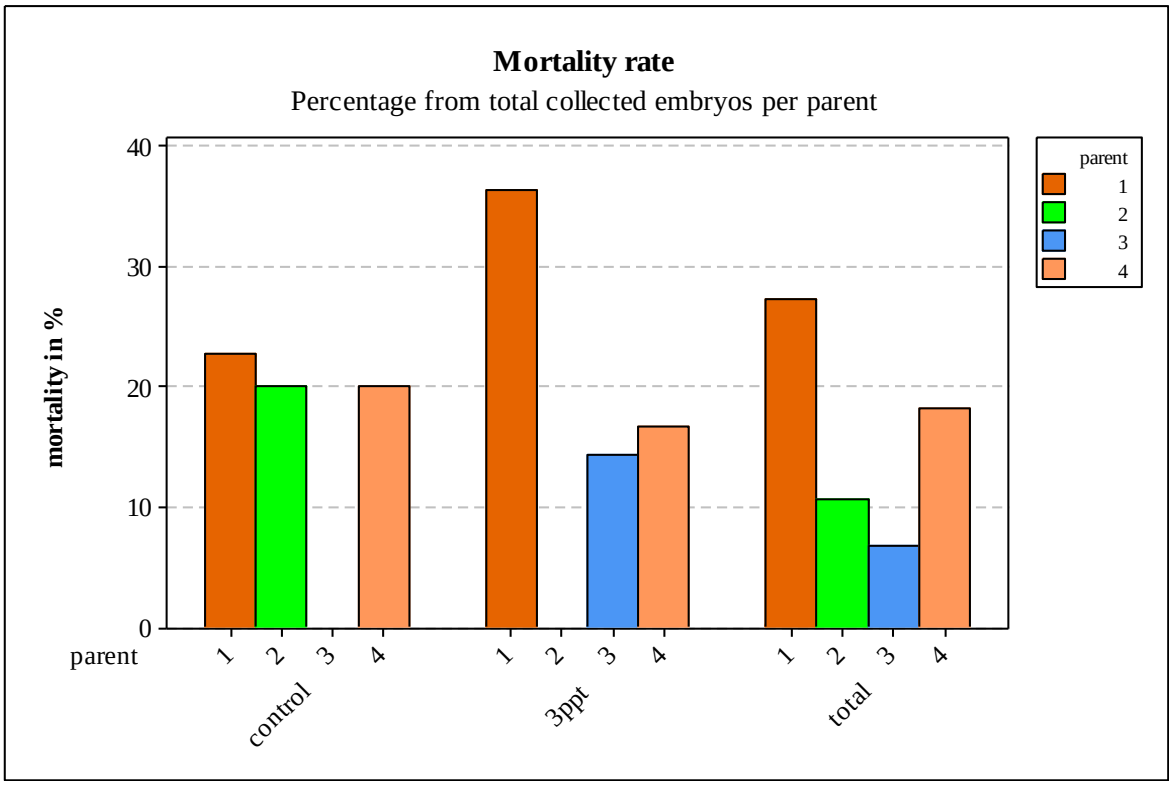

Figure 17: Percentage of embryos that died per parent by treatment (control and 3ppt) and for the total of collected embryos for each parent

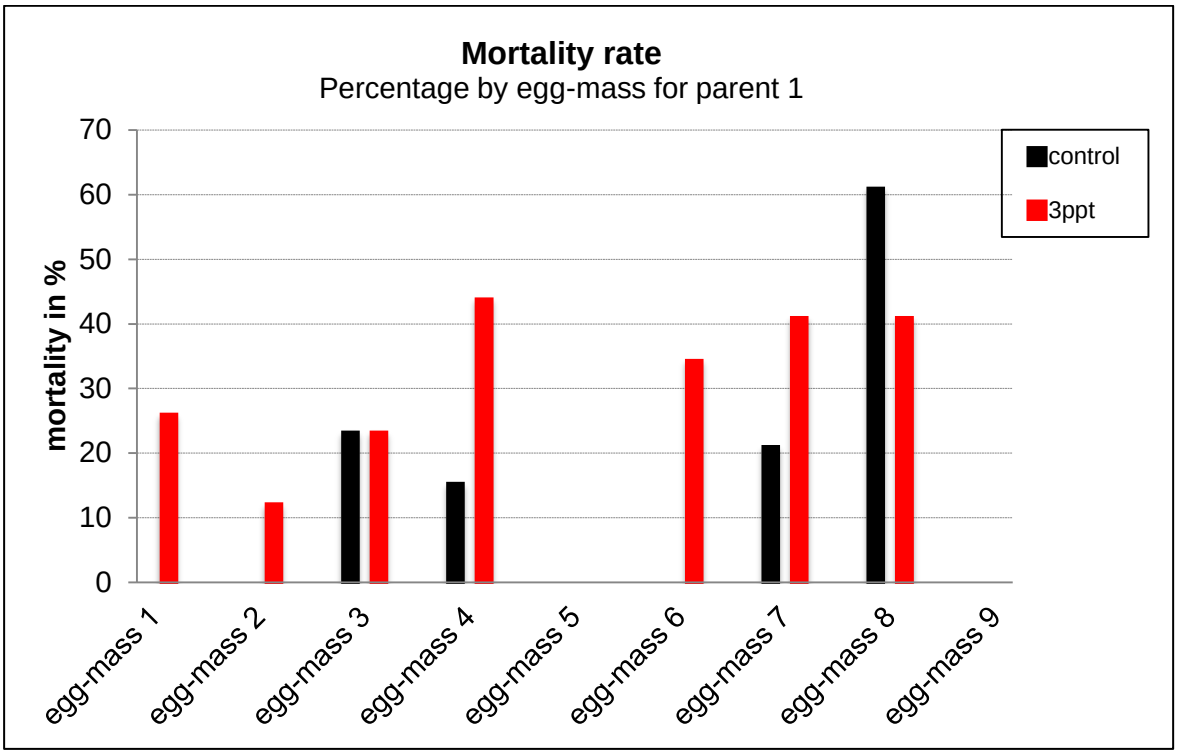

Figure 18: Percentage of embryos that died during the development per egg-mass by treatment (control and 3ppt) for parent 1

Discussion

In this study we investigated the relative importance of genetic similarity by parentage and egg-mass origin versus the importance of environment variation on intra-specific heterochrony in *Radix balthica*.

The data obtained from embryos that survived post-hatching was treated separately from the data obtained from embryos that died during their development to avoid diminishing possible observed effects.

The variation in the sequence of developmental events appeared to be a response to the environment to which the embryo is exposed. Variation in different parents and egg-masses was found in the control treatment alone (parent 3; egg-masse 1, 2 and 5); in the increased salinity alone (parent 2) and in both treatments together (parent 1 and 4; egg-masse 3 and 4). This suggests some parental and egg-mass adaptation to a specific environment.

Mortality rates varied from 0% to 35% in the increased salinity treatment and from 0% to 20% in the control treatment. This shows great variability in the mortality level between environments. Embryos that originated from parent 1 and parent 4 showed relatively similar mortality levels in both treatments whereas embryos that originated from parent 2 showed high mortality levels in the control treatment and no mortality in the increased salinity treatment. Mortality levels in embryos from parent 3 however were the opposite of those in parent 2, no mortality in the control treatment and

elevated mortality in the increased salinity environment. Mortality within parent 1 showed that overall embryos have a higher mortality rate in the increased salinity treatment than they do in the control treatment. A similar pattern was found between egg-masses of parent 1.

When comparing the level of mortality to the variation in sequence a strong correlation was found at both parent and egg-mass level. The parents and egg-masses showing variation in the sequence of development in a particular treatment showed nil mortality in that same treatment. The parents and egg-masses showing variation in the sequence of development in both treatments show mortality in both treatments as well. This is strong evidence that the variation in the sequence of developmental events undergone by embryos in certain treatments allows them to better adjust to that treatment and therefore lowers the mortality observed in that particular treatment. The variation in the sequence of developmental events appears to be a plastic response depending on the environment to which the embryo is exposed. The better adjustment of embryos originating from parent 2 and 3 to a specific environment could be an indication of speciation by environmental adaptation though sequence heterochrony with parent 1 and 4 being intermediate forms and producing offspring adapted to a range of environments.

The embryos that survived post-hatching showed no significant variation early in their ontogeny. These findings correspond to the findings by Smithwaite *et al.* [9] who found variation in the development from mid to late ontogeny. All five developmental events recorded after the appearance of eye spots showed significant variation in the timing of embryonic development depending on parental origin. The results from this study showed that the intra-individual variation in the developmental timing of *R. balthica* embryos is mostly influenced by the parental origin. The observations support the findings by Tills *et al*. [16, 17] who found parent-offspring similarities in the developmental timing. The results showed that the environment had no effect on the timing of development in *R. balthic.* Hatching was the only event found to be influenced by both the parental origin and the treatment to which the embryos were exposed, indicating the timing of hatching is influenced by both a genetic component and environmental plasticity. The significance of egg-mass origin in the appearance of the heart suggests a potential similarity in the development between embryos that originated from the same egg-mass in the early developmental events that show heterochrony in the basommatophoran group. However this potential influence needs further investigating with larger egg-masses than were used in this study as the small size of some egg-masses in this study might hide the full the extent to which egg-mass has an effect on the development of *R. balthica*. Foot attachment and crawling were the two events previously found to show heritability [16]. These two events were found to be influenced by egg-mass origin and parentage; these findings are similar to the findings by Tills *et al*. [16].

No factor was found to affect daily heart rate of embryos that survived post-hatching. A stronger stressor might reveal effects that were not found in this study.

Conclusion

This study has shown that intra-specific heterochrony in influenced by both parentage (i.e.: it has a genetic basis) and by the environment to which the embryo

is exposed to during its ontogeny (i.e.: environmental plasticity). However, the influence of genetics and the plasticity on the developmental variation in *Radix balthica* is different. The genetic component of the variation was found to affect only the timing of the developmental events. Except for the timing of hatching, only the genetics were found to affect the timing of the developmental events. On the other hand, the environment was only found to influence the sequence of developmental events and only the timing of hatching. It is unclear from this study to what level the genetics or the environment influences the timing of hatching. Further investigations are needed to understand to what extend the findings of this study apply to other taxa and how multiple environmental factors interacting are important in driving intra-specific heterochrony compared to genetics. Carrying out a similar study between closely related species could provide the missing link between ontogeny and phylogeny if both the genetics and environmental plasticity are found to drive inter-specific heterochrony in the same way they were found to drive intra-specific heterochrony in this study.

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