Geographical Variation in Life History Response to Stress in the Freshwater Snail, *Lymnaea stagnalis*

by

Iain Grant Reid

A thesis submitted in partial fulfilment of the requirements of Edinburgh Napier University for the award of Doctor of Philosophy

School of Life Sport and Social Sciences August 2013

“We have to continually be jumping off cliffs
and developing our wings on the way down”

Attributed to Kurt Vonnegut

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Abstract

Life histories are known to vary across geographic ranges in response to a number of factors, both biotic and abiotic. Environmental calcium availability has been shown to affect freshwater gastropod life histories due to its fundamental requirement in shell formation. Adaptation of life histories to local environmental conditions may cause the response to novel pollutants to vary across populations within a species due to trade-offs between and among traits but very few studies have examined wide scale variation in life history response to stress across geographic ranges.

A long term study was conducted and aimed to expand on current understanding by rearing populations of the great pond snail, *Lymnaea stagnalis* sampled from across the UK, for two generations in high and low calcium environments. A comprehensive suite of life histories was recorded throughout the study and traits were compared between populations and treatments, and across generations to distinguish between environmental, plastic and heritable sources of variation. Further work focussed on life history trade-offs under different environmental conditions before utilising a stage-classified matrix model to derive population growth rates (λ) and compare effects across populations and calcium treatments. Finally, acute and chronic effects of exposure to nanoparticulate carbon black were assessed before using matrix models to investigate the combined effects of environmental calcium with nanoparticulate carbon black on λ for three populations of snails.

Significant intra-specific variation was recorded in the majority of life history traits, which were shown to display high levels of phenotypic plasticity as the norm. Intergenerational comparisons revealed that traits more directly linked to fitness, such as size at reproduction and reproductive output, showed higher heritabilities than those pertaining to growth, such as growth rates and age at first reproduction. Both generalised and population specific responses to calcium availability were shown in life history traits across the study populations. These effects tended to be subtle but suggest that environmental calcium plays a role in shaping life history strategies across the UK distribution.

Life history trade-offs between traits tended to be conserved across populations, and showed little response to environmental calcium, although differential investment in life history traits across calcium treatment was detected in some cases. A strong trade-off between age and size at first reproduction was detected across all generations and calcium treatments. Size at first reproduction was also shown to correlate with reproductive output, wherein a trade-off between eggs per mass and number of egg masses was detected. Traits involved in trade-offs appeared to more strongly associate with fitness and these findings suggest that trade-offs between key life history traits are of importance in understanding population specific life history strategies. Stage-classified matrix modelling showed a trend towards reduced λ in low calcium but this trend was not significant. A significant reduction in λ across generations was recorded which was most likely to be the result of inbreeding.

Local adaptation to calcium availability was shown to influence the life history response to nanoparticulate carbon black, and was mirrored in predicted population growth rates obtained from matrix models. Intraspecific differences in response to carbon black nanoparticles only became apparent when calcium concentrations were low. These findings would support the view that in order to be better able to predict the response of species to the presence of novel stressors such as nanoparticles, it is necessary to account

for intraspecific adaptation of life history traits as well as geographical variation in the environmental context.

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1. Introduction and literature review

1.1. Life history studies:

From birth, through life, to death, each and every organism will exhibit a unique response to the challenge of living. This response describes the organisms' life history. Stearns (1992) states that such life histories are characterised by variation in principal traits such as:

1.) Size at birth
2.) Age at maturity
3.) Size at maturity
4.) Number, size and sex ratio of offspring
5.) Age- and size-specific reproductive investments
6.) Age- and size-specific mortality schedules
7.) Length of life

Central to current life history theory is the view that the life history strategy employed by a given organism is subject to natural selection in order to ensure maximal fitness (here defined as the number of offspring surviving to contribute to the next generation) (Stearns, 1980).

Life history strategies vary across the biological world. Traits pertaining to reproduction and growth are most likely to be subject to the strongest levels of selection, and thus r/K selection theory can be used to interpret much of life history variation (Begon et al., 2006). r-selected organisms tend to be short lived, with rapid growth, high reproduction and low parental investment. For example mayflies, representing the extreme end of r-selection, are *semelparous*, being short lived, producing many offspring in one reproductive event before dying, with no parental care being delivered to the offspring. r-selected strategists tend to be found in unstable environments (for example in polluted sites or in seasonal environments) where rapid growth and high reproduction compensate for high juvenile mortality. By contrast, K-selected organisms tend to be longer lived, producing fewer offspring over a longer time period, with greater parental care. K-selected organisms, such as humans, tend to be *iteroparous*, and are typically found in more stable

environments, being capable of producing multiple offspring over a long lifespan, with low levels of juvenile mortality (Stearns, 1992).

The use of r and K classification serves as a crude, but still useful, way of interpreting the continuum of life histories observed within the natural world. In reality an organism's life history strategy is a result of the interaction of multiple environmental (e.g. parasitism, pollution or temperature) and genetic (genotypic variation and natural selection) factors over evolutionary time and not dictated solely by growth and reproduction.

1.1.1. Demography and life tables

Demography is the study of population dynamics with respect to age structure and natural selection (Stearns, 1992). Initial demographic studies focussed on human populations with the intention of deriving a means of projecting population growth (Begon et al., 2006). Such forecasts were made possible by collection and analysis of human life history data, comprising an account of generational birth and death rates, typically expressed in the form of a life table (Stearns, 1992).

Life tables originated from studies of human demography, where recording and monitoring of birth and death rates were used to calculate the probability of individuals surviving to a particular age and their remaining life expectancy (Begon et al., 2006). Life tables are typically one of two forms; *static* or *period* life tables and *cohort* life tables. Cohort life tables are more difficult to construct as they involve following a cohort of individuals from birth to death, recording all reproduction and mortality as it occurs. Static life tables are constructed from a mixture of cohorts, considered representative of all age classes at any given time, and assume no year to year variation in mortality or reproduction.

Life tables are used to calculate some of the basic metrics of population growth. Some common parameters used to describe population demography are displayed below in Table 1.1.

Table 1.1. Common parameters used in population demography (adapted from Stearns, 1992).

Symbol(s)	**Definition**
λ, e^r	Rate of population growth per unit time
R	Intrinsic rate of increase
R_o	Lifetime reproductive rate
X	Age
X	Age interval/class
T	Time
S_x	Number surviving to age class x
D_x	Number dying between age class x to x + 1
$q_x = D_x / S_x$	Finite rate of mortality between x to x + 1

In population ecology perhaps the two most important descriptive metrics are the intrinsic rate of increase, r, and the rate of population growth per unit time, λ. These metrics are calculated from existing population sizes and use birth and death rates to indicate whether the population is growing, stable or in decline. The intrinsic growth rate, r, is the difference between the *per capita* birth rate and death rate, and as such, a value of r greater than 0 indicate population growth, a negative value indicates that the population is in decline and a value of 0 indicates that the population is stable (where birth rates equal death rates). The value of λ can either be approximated directly from r (in the form $\lambda = e^r$) or from the ratio of number of individuals in the population before and after a given time step, T (typically years, but it depends on the specific organism or case study). A λ greater than 1 indicates population growth, while values less than 1 indicate population decline. Populations with λ values equal to 1 are assumed to exist in a stable state. Although under certain circumstances λ and r may be used interchangeably to describe population growth, they are not the same and tend to diverge more as growth rates and time steps become larger (Begon et al., 2006).

1.1.2. Matrix modelling in life history studies

Matrix population models were initially developed by Leslie (1945) as a way of projecting life table data and allowing population growth metrics to be calculated over relevant timescales (Caswell, 2000). Models can be constructed using data derived from specific age or stage classes (Caswell,

2009, Sandrine et al., 2009). By gathering a suite of data from a number of individual organisms, a set of stage or age specific *survivorship* probabilities (*Pi*), *transition* probabilities (*Gi*), and *fecundities* (*Fi*), in this context expressed as reproductive output per individual per time step, can be used as inputs to a matrix model that may then be employed to generate a range of numerical end points that can be used to classify the populations under study, by using key parameters such as the population growth rate, λ, which is the dominant eigenvector of the matrix (Caswell, 2000).

Further *elasticity* analysis can be performed to assess the relative contribution from each stage or age to the calculated λ values (Caswell, 2000). As such, matrix models are a useful tool for forecasting population growth, for dissecting and analysing patterns of growth and constraints, and may be used in a wide range of applied aspects, from examining the natural population structure of killer whales (Brault and Caswell, 1993) to that of endangered turtles (Enneson and Litzgus, 2008), to assessing the impacts of environmental pollutants in ecotoxicological studies (Billoir et al., 2007, Sandrine et al., 2009), or the effects of overfishing in commercial crab species (Miller, 2001).

1.1.3. Mechanisms of life history evolution: Genetic variation, phenotypic plasticity & reaction norms

Variation, the underlying raw material on which natural selection acts, is itself constrained by both the phenotype and genotype (Stearns, 1989a). The inherited information of the genotype is 'hard wired' at birth, and within the inherited genotype lies information allowing the organism to respond to a number of hypothetical 'environments' (Stearns, 1989a). Such response to variable abiotic (or biotic) conditions during life is termed *phenotypic plasticity* and can result in measurable phenotypic differences in the same genotype when it is reared in different environments (Scheiner, 1993). Genetic variation around a particular trait may also result in measurably different phenotypes (as a direct result of different genotypes) being observed and care must be taken to distinguish whether the observed effects are genetic or phenotypically plastic in origin (West-Eberhard, 1989).

Thus, observed differences between phenotypes may result from a combination of both genetic and genotype with environment interactions. Phenotypic plasticity can mask genetic differences and it is possible for different genotypes to present the same phenotype in one environment but differ in their phenotypic response in others, (Stearns, 1989a, Stearns and Koella, 1986).

When a clear relationship exists between environmental conditions, a single genotype and the observed phenotype, the response is termed the *reaction norm* (Stearns, 1992) and a schematic representation of this relationship is displayed in Figure 1.1 Genetic changes rarely result in variation in one aspect of the phenotype and *pleiotropy* occurs where a single genetic change results in differences in multiple measurable phenotypic traits (Stearns, 1989b).

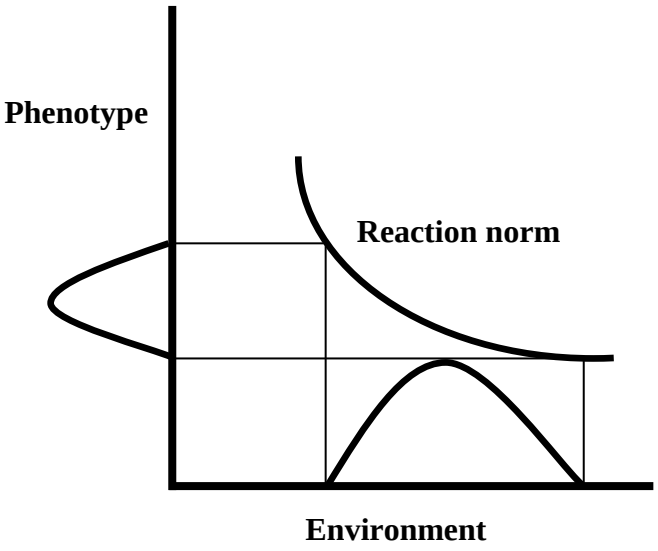


Figure 1.1. Schematic representation of the relationship between the environment and the observed phenotype; whereby environmental differences result in different phenotypes being observed. Adapted from Stearns (1989a).

Where more than one genotype is exposed to different environments the reaction norms may run parallel to one another (i.e. all genotypes respond in the same way to environmental differences), or their slopes may differ,

indicating the presence of genotype x environment (G x E) interactions, where differences between phenotypes vary across the environments they are found in. Such G x E interaction is displayed in Figure 1.2. whereby genotypes 1 and 2 result in measurably different phenotypes only in environments 1 and 3, with genetic differences being masked by phenotypic plasticity in environment 2, where the reaction norms cross (Stearns, 1992).

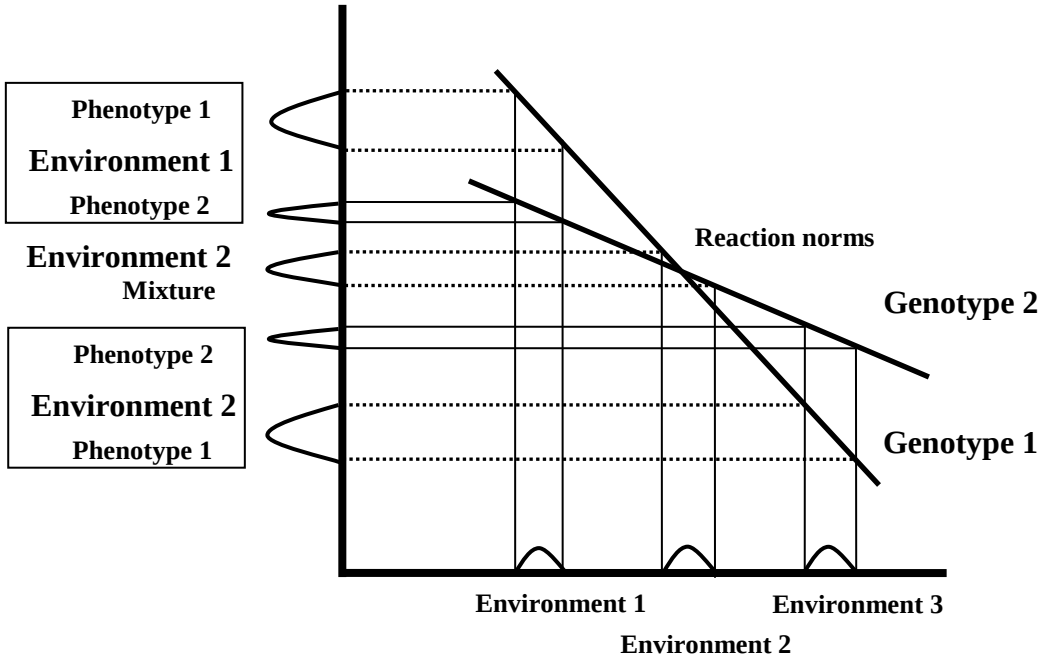


Figure 1.2. Schematic representation of the relationship between phenotypes derived from two genotypes reared in multiple environments. No difference between phenotypes is detected where reaction norms cross, as occurs in environment 2. Adapted from Stearns (1992).

Thus the effects of genetic variation, combined with phenotypic plasticity and varying environmental conditions, may result in a number (effectively a continuum) of measurably different phenotypes being recorded for different populations of any given species over a geographic range. Ultimately, the range of possible G x E interactions is constrained at the genetic level and the continuum of phenotypes observed is not limitless, being constrained by these factors (Stearns, 1989a, DeWitt et al., 1998).

In much the same way as individual traits, the evolution of phenotypic plasticity is also subject to costs and limits (Auld et al., 2010, DeWitt et al., 1998). Costs include *maintenance costs*, the energetic costs of maintaining

sensory and regulatory mechanisms to allow plasticity in the first place; to *information acquisition costs*, associated with the expression of phenotypes inappropriate to the environment (Auld et al., 2010, DeWitt et al., 1998). Limits to plasticity include *developmental range limits* where plastic phenotypes lack the ability to deliver more extreme phenotypes that genetically fixed types. *Lag-time limits* result from phenotypic changes that require time and result in sub optimal phenotypes and a temporal reduction in fitness.

Life history models and theory predict that phenotypic plasticity itself should be subject to selection and should be greatest in organisms that evolve in a changing and dynamic environment and be less pronounced in those that evolve in relatively stable environments (Stearns, 1989a). This has been found by Siegel and Ford (2001) who demonstrated that phenotypic plasticity in clutch size and mass varied in different populations of Checkered Garter Snakes, *Thamnophis marcianus*, across a geographic range and similarly by Lardies and Bozinovic (2008) who demonstrated that phenotypic plasticity in the terrestrial isopod *Porcellio laevis* was shown to vary in response to latitude (and therefore environmental heterogeneity) . These authors suggested that environmental heterogeneity (in the form of local variability in food or environmental conditions) may ultimately have been responsible for canalising or increasing the plasticity of traits over time in the different populations observed.

1.1.4. Trade-offs:

Heritable variability in any one of the above life history traits will produce variation in phenotypic character that (subject to natural selection) may then be selected for on the basis of fitness, i.e. the number of surviving offspring that contribute to the next generation (Stearns, 1992). Central to life history theory is the view that the energy available to any organism is finite, and there are limitations imposed on the energy allocated to other life history traits when another is selected for (Stearns, 1989b). Such limitations are termed ‘trade-offs’ and are constrained by both the genotype and phenotype of the given

organism in response to its specific environment (Brown, 1979b, Reznick, 1983).

Stearns (1992) draws the distinction between phenotypic (*physiological*), and genotypic (*microevolutionary* and *macroevolutionary*) trade-offs. Physiological trade-offs are those that take place in resource allocation between two or more biological processes (such as maintenance and growth vs. reproduction as described in red deer, *Cervus elaphus* by Clutton- Brock *et al* (1983)) within an individual organism during its life. Microevolutionary trade-offs typically involve physiological trade-offs and take place at the population level, where changes in one trait pertaining to increased fitness bring about changes in another trait. In order for microevolutionary trade-offs to occur the physiological trade-off must be selected for and those physiological trade-offs that are purely a result of phenotypic plasticity (such as many ectotherm growth responses to temperature) with no underlying genetic variation will not result in microevolutionary trade-offs. Macroevolutionary trade-offs occur above the species level and typically involve negative correlations across traits that are fixed at the individual species level (Stearns, 1992).

Forty-five trade-offs between life history traits are described by Stearns (1992) who suggests that many more are still to be found. Trade-offs tend to be measured experimentally as (typically negative) correlations between one trait (e.g. number of eggs produced) against another (e.g. egg size) and may be readily detected in field studies across different levels of biological organisation (between (macroevolutionary trade-offs) and within species (microevolutionary and physiological trade-offs)), and in laboratory based experiments (physiological and some microevolutionary trade-offs), where typically one trait is manipulated (Begon et al., 2006). The most commonly reported trade-offs in the literature relate to key life history traits directly involving growth, reproduction and survival (Reznick, 1983, Roff et al., 2002, Stearns, 1989b).

Stearns (1989b) reports that the cost of reproduction (CR) is central to the understanding of life history trade-offs. The costs of reproduction can effectively be split between those that affect immediate survival and those that affect future reproductive effort (Begon et al., 2006). An organism that invests heavily in current reproduction is likely to reduce its capacity for future survival, and as such key life history characteristics such as the timing of, and size at first reproduction, are likely to display strong trade-offs with other traits such as growth, body condition, and survival. As with adult size at reproduction, the number and size of the offspring produced is expected to influence fitness in both the adults and offspring, this relationship first examined in seminal studies of clutch size in birds by Lack (1947). Trade-offs may extend beyond the organism itself and involve interaction with external environmental factors such as temperature and biological interactions such as predation and parasitism (Rigby and Jokela, 2000, Trussell, 2000, Trussell and Nicklin, 2002).

1.1.5. Intra-specific adaptation

Abiotic conditions fluctuate considerably across the geographic range that any given species inhabits (Gaston, 2003). For example, variation in temperature (and temperature range) occurs across latitudinal gradients, where temperature generally declines with distance from the equator to the poles, while temperature range increases with latitude. In addition to temperature, local availability of macronutrients may limit productivity for primary producers (Schindler, 1977) (and therefore food supply for heterotrophs) while physico-chemical characteristics (e.g. such as pH, conductivity and oxygen concentrations in fresh waters) may vary over large and small scales (Townsend et al., 1997).

Such environmental heterogeneity, combined with physical restrictions in gene flow (i.e. due to limited dispersal activity or physical barriers), may result in differential selection over the species range. This selection may result in intra-specific adaptation of life history characteristics at any given locality (Lam and Calow, 1989a, Lam and Calow, 1989b, Telfer and Hassall, 1999, Olsson and Agren, 2002). A study by Cardoso and Defeo (2004)

demonstrated the typical pattern of declining size across the latitudinal range of the isopod *Excirolana braziliensis,* with the smallest individuals occurring at tropical beaches (9°N) and the largest being found at temperate sites (39°S). Adaptation is likely to be most pronounced in those populations found at range margins where abiotic conditions may be limiting (Hassall et al., 2006). Under such limiting abiotic conditions, adaptation will occur until it is constrained by a combination of trade-offs with other traits and the extent of underlying genetic variation. At this point no further adaptation is possible, limiting the expansion of distribution over geographic areas. In these circumstances, due to the extent of trade-offs necessary to maintain their existence, species may be less able to adapt to the effects of other stresses, such as anthropogenic pollution, and hence be more vulnerable to the impacts of these factors. The implication of this is that the response of a species to a given environmental perturbation such as a toxic chemical is likely to vary depending on the population source and environmental context (Medina et al., 2007), and may show broad geographic patterns of variation in response linked to limiting abiotic conditions. Studies such as Baird and Van Den Brink (2007) have demonstrated that the responses of organisms to pollution depended on the origin of the test population. These results are likely to be due to different adaptation of life history of the different populations studied. Previous studies have examined patterns over a small spatial scale while larger scale studies, where variation may be even more prevalent, are yet to be undertaken.

1.1.6. *Lymnaea stagnalis*: Biology and ecology

Lymnaea stagnalis is a freshwater pulmonate gastropod mollusc (Boycott, 1936). Pulmonate snails are evolutionary descendants of solely terrestrial ancestors that retain lungs that are filled through air contact at the water interface via a contractile opening called the *pneumostome* (McMahon, 1983). Pulmonates are bimodal breathers, being also capable of respiration through the skin and *L. stagnalis* is capable of surviving solely via cutaneous respiration when temperature and oxygen conditions are favourable (McMahon, 1983); typically *L. stagnalis* cease to ventilate the lung below 10^{o}C (Sidorov, 2005). *L. stagnalis* are truly iteroparous members of the family

Lymnaediae where reproduction is continuous following sexual maturity and the adults do not die after reproduction, living to an age of up to two to five years (Calow, 1983, Russel-Hunter, 1964). *L. stagnalis* are simultaneous hermaphrodites, possessing both male and female sexual function (Koene, 2006). Parthenogenic reproduction is also possible, although *L. stagnalis* show a tendency to avoid selfing in favour of sexual reproduction where possible (McMahon, 1983). Although *L. stagnalis* can mate as both male and female, mating is only carried out in one sexual role at a given time. When mating as males *L. stagnalis* typically circle round another snail in a counter clockwise manner with the *preputium* (a retractable organ which contains the penis) everted until the penis is inserted into the other individual and fertilisation takes place (Koene, 2006). When mating in the female role, *L. stagnalis* remain stationary while the preputium is inserted, whereupon a large amount of ejaculate is received and can be stored to be used to fertilise eggs at a later date (Koene, 2006). The energetics of male and female sexual function are unequal and initial copulations in the male or female role result in different fecundity and growth for individuals acting as predominantly male or female (Koene, 2006, Koene and Ter Maat, 2004).

Cylindrical egg masses of generally between 40 and 120 eggs are laid encapsulated by a gelatinous membrane, the *ootheca* (Wagner, 2000). Embryos develop as miniature adults and hatch 2-4 weeks after being laid. Juvenile *L. stagnalis* spend a longer time out the water when compared to the adults (Gerard et al., 2005). Growth is rapid, with animals held at temperatures of 20^{o}C becoming sexually mature at a size of 10-20mm within 3-6 months, with oviposition rates of 0.2 capsules per snail per day, and attaining a maximum shell size of 50-60mm after several years (Van Der Steen et al., 1969, Van Duivenboden et al., 1985).

The UK distribution of *L. stagnalis* is shown in Figure 1.3. The distribution of *L. stagnalis* is believed to be largely limited by calcium (Ca) availability due to the fundamental physiological requirement of this nutrient in the construction of the shell (Briers, 2003, Boycott, 1936). *L. stagnalis* are considered to

require high environmental calcium concentrations relative to other freshwater molluscs and as such is described as a *calciphile* species by Boycott (1936)

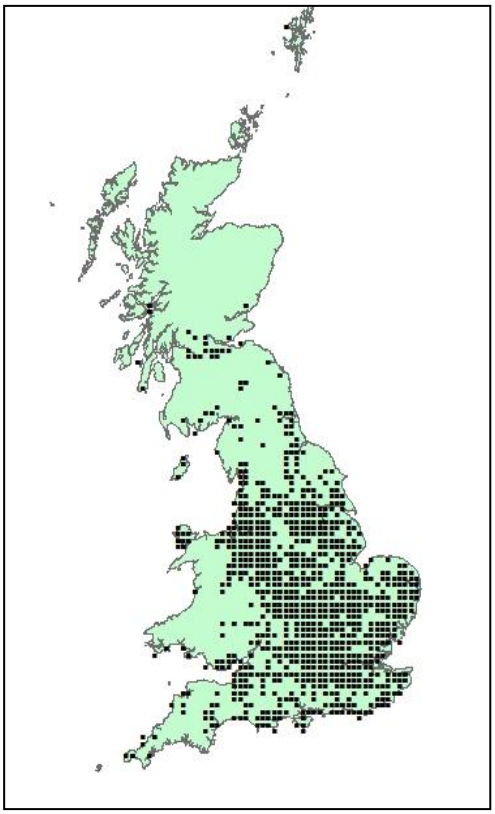


Figure 1.3. UK distribution of *Lymnaea stagnalis*. Black squares indicate 10km^2 tetrads where *L. stagnalis* has been recorded since 1970. (Country border data © Crown Copyright/database right 2008. An Ordnance Survey/EDINA supplied service. *L. stagnalis* distribution data provided with permission of the National Biodiversity Network.)

The energetic costs involved in Ca sequestration from the environment are high (McMahon, 1983) and hence adaptation of life history to low calcium conditions may result in a trade-off in favour or against the ability to respond to other environmental variables. The range margins of *L. stagnalis* distribution (towards the west and north) correspond to areas of low Ca availability and as such it can be hypothesised that in order to survive and reproduce, populations in these areas may display local adaptation of physiology and life history to compensate for this reduced Ca availability, although other factors such as changes in temperature regime are also likely to play a role in limiting distribution.

1.1.7. The suitability of molluscs in ecotoxicological studies

A wide range of molluscs have been utilised as test organisms in ecotoxicological studies (Rittschof and McClellan-Green, 2005). Pulmonate gastropods, such as *L. stagnalis* have been shown to accumulate heavy metals and tend to be more tolerant of water pollution than other taxa, but there is substantial variation in the sensitivity of different life stages (juvenile/embryonic versus adult). (Wagner, 2000). Various pollutants such as endocrine modulating substances (Czech et al., 2001, Leung et al., 2007), metals (Elangovan et al., 1997, Coeurdassier et al., 2003), insecticides (Presing, 1993) and organic enrichment (Wagner, 2000) have been shown to impact *L. stagnalis* life history. Studies by Salanki *et al* (2003) used video capture and movement analysis to show that metal contamination caused changes in the orientation and geotaxis of *L. stagnalis* and Coeurdassier et al. (2003) noted changes in growth, reproduction and hatching success in relation to cadmium exposure. The ability of molluscs to accumulate toxic substances (most likely via ingestion of contaminated sedimentary plant material) makes them ideal to assess the effects of chronic exposure to particulate pollutants and the effects that these may have on life history traits.

The focus of this research project therefore lies in establishing the degree of intra-specific variation in life history response to environmental variation across the geographic range of *L. stagnalis*. The initial focus will be on adaptation to different environmental calcium levels, before considering combined effects of environmental context and exposure to a novel anthropogenic stressor, namely nanomaterials.

1.2. Nanotechnology

In the 1960s the Nobel prize-winning physicist Richard Feynman proclaimed ‘There’s plenty of room at the bottom’ when he foresaw the emergent possibilities of the ever diminishing size scale of technologies (Whatmore, 2006). Feynman went on to predict the changes in materials technology and manufacture that have given rise to the boom in nanotechnology. The late twentieth century heralded an expansion in the nanotechnology industry with worldwide investment in nanotechnology research estimated to rise to $1

trillion by 2015 (Wilson, 2006). Engineered nanomaterials (NMs) can be found in a wide variety of common products, ranging from clothing, to bowling ball coatings, and fuel cells (Colvin, 2004). Expanding fields such as nanomedicine, where the development and use of nanomaterials such as cadmium-telluride (CdTe) nanocrystals for medical imaging and nanotubes for therapeutic (drug delivery) purposes have considerable clinical and economic potential (Kagan et al., 2005). NMs are being developed, and some are already in use, in the areas of waste water management and bioremediation of polluted environments, such as the use of nanoscale iron particles to detoxify and transform chlorinated organic solvents and Polychlorinated biphenyls (PCBs), which will involve the direct intentional release of these materials into the aquatic or terrestrial environment (Bottero et al., 2006, Zhang, 2003). While the nanotechnology industries enjoy exponential growth there remain concerns as to the human and environmental risks that may be associated with NMs (Wilson, 2006). There is therefore a need to understand the long and short term implications of NMs, both in terms of the potential impacts on human and environmental health, that their unregulated proliferation in the environment may have (Colvin, 2004, Moore, 2006, Wilson, 2006).

1.2.1. Nanomaterials: Definition and properties

Colvin (2004) defined nanoparticles as those having at least one dimension below 100 nm. There have been more recent debates in this area with the European Commission and ISO providing slightly different definitions. The debate is ongoing but in the context of this study the EC nomenclature is followed, with nanomaterials (NMs) considered any particulate matter with at least one dimension at the nanoscale (between 1-100 nm) and nanoparticles (NPs) any particulate matter with three dimensions within the nanoscale. A nanometre is one-billionth of a meter and equates to a dimension approximately 10 atoms in width (Whatmore, 2006). Much nanosafety research was based on earlier studies of air pollution which introduced the term ultrafine particles (UFPs), which were defined by Oberdorster *et al* (2005) as those with a diameter less than 100nm and are typically airborne. For consistency in this thesis all nano-sized particles will herein be referred to

as NMs. Characteristic of all NMs is the increase of surface area to volume ratios associated with a reduction in size to the nanoscale (Whatmore, 2006, Wilson, 2006). The increase in surface area to volume ratio confers an increase in surface reactivity of the NMs relative to the parent or bulk material (larger than 100 nm) and thus a common response of biological systems to nanoparticle exposure is increased oxidative stress (Oberdorster et al., 2005, Wang et al., 2009). Nanosize materials also display different optical, magnetic and electrical behaviour from their parent bulk materials, indeed, some nanomaterials are so small that they may be influenced by atomic forces such as van der Waals forces (Whatmore, 2006). It should be noted that naturally occurring NMs, such as biogenic magnetite or carbon particulates (such as fullerenes) from forest fires or volcanoes, are found throughout the natural world. In comparison to naturally occurring NMs which have been present throughout the process of evolution, anthropogenic NMs represent a novel class of materials that vary greatly in their composition, surface coatings, and therefore reactive, properties, both in their raw and functionalised forms. At present, despite a growing number of studies there is still insufficient knowledge of their toxicology or resultant environmental fate to be sure of their potential impacts (Moore, 2006, Oberdorster et al., 2005).

In order to understand the potential threat posed by NMs it is first necessary to recognise that both particle size and surface reactive properties may contribute to any toxicological effects on a given organism. Model NMs of relatively inert, non-toxic parent material such as titanium dioxide (TiO_2), used for sunscreens and paint pigments, and carbon black (CB), used commonly as printer dust and in tyre manufacture, have been shown to induce lung inflammation in rats where larger non-nanosized particles of the same material do not (Rosenkranz et al., 2009, Li et al., 1999, Ferin et al., 1992). Toxicity derived specifically from mechanical action, or structural features of NMs becomes apparent when considering carbon nanotubes, which due to similarities in size and structure to asbestos filaments, could be damaging to respiratory function leading to the development of pulmonary fibrosis and cancer (Hoet et al., 2004). As such, NMs present a new challenge in the field of toxicological understanding, whereby the mechanical and chemical

properties of the substance, the subsequent interactions that they have with biological systems, and the chemical transformation that this may infer, require elucidation in order to fully understand the nature of the threats that they may pose.

1.2.2. Environmental fate of nanomaterials

Besides the intentional release on NMs for bioremediation purposes, there are also risks from the accidental, but potentially large scale, release of nanomaterials into the aquatic environment. The hydrophobic and lipophilic surface properties of many NMs make them likely candidates for bioaccumulation and bioconcentration in organisms and food chains (Moore, 2006). Due to their particulate nature it is likely that many NMs in aquatic systems will sink and may be incorporated within sediments due to their high surface area to volume ratio (Oberdorster et al., 2005). Moore (2006) goes on to suggest that dissolved colloidal substances with hydrophobic character such as humic acids in freshwaters may bind with NMs rendering them more dispersed in water which may lead to enhanced exposure and thus toxicity. Routes of entry into living systems include respiratory, ingestion and through the skin (Oberdorster et al., 2005, Klaine et al., 2008). These authors go on to state that NMs may then enter the vascular system and translocate and accumulate in specific organs and cells (via endocytotic pathways). In higher organisms translocation of inhaled radioactive NPs from the lungs, via the blood, to the bladder has been shown to occur (Hoet et al., 2004). Transformation, through degradation by chemical and biological agents and UV light, of released NMs is also likely to be a cause for concern as this infers the possible activation of inert NMs to bioreactive and toxic types (Moore, 2006, Zhang, 2003). There is therefore a pressing need to assess both the direct toxicity of released NMs and the long term chronic effects of release into the aquatic environment and the effects that this may have on overall ecosystem integrity (Moore, 2006, Oberdorster et al., 2005, Whatmore, 2006).

1.2.3. Nanotoxicology and nanoecotoxicology

The growth of the nanotechnology industry, coupled with a lack of knowledge associated with NM toxicology and the potential for unchecked release of

NMs into the environment, has brought about the emergence of the subdiscipline of nanotoxicology (Fischer and Chan, 2007). Although there is now more than a decade of work that has started to explore and document effects of NMs, nanotoxicology still remains in its infancy (Moore, 2006). To date, there is still much lack of data with regards to the effect of NMs in the environment as well as lack of knowledge regarding their fate and behaviour in the freshwater environment.

Within the increasing number of ecotoxicological studies that have been carried out on different NMs, the majority of data are derived from aquatic organisms. However studies conducted focus on a few model systems such as *Daphnia* (Kahru and Dubourguier, 2010) and there remains a paucity of data relating to the effects and fate in freshwater environments. There is currently much debate regarding the appropriateness of standard protocols for hazard assessment of NMs (Handy et al., 2012a, Handy et al., 2012b). Intrinsically NMs are particulates and as such do not tend to disperse or dissolve in aquatic systems. The nature of NM fate will obviously depend on the particles themselves, including their source material, surface coatings, etc, with some having a hydrophobic and lipophilic character (Oberdorster et al., 2006). Attempts to disperse the fullerene C_{60} throughout exposure media by Oberdorster et al (2006) included employing sonication, stirring for prolonged periods and the use of tetrahydrofuran (THF) as a carrier solvent. These authors and a similar study by Lovern and Klaper (2006) indicated that the use of THF and sonicated C_{60} brought about increased mortality in the test species compared with water alone. Studies by Henry et al (2007) clearly demonstrated that the toxic effects of C60 suspended in THF were due to a THF degradation product (γ-butyrolactone) rather than to C60, which may explain the toxicity attributed to C60 in other investigations.

The insolubility of NSPs makes the need to deliver a specific quantifiable dose in aquatic toxicology extremely difficult. It is pertinent to ask whether the sonication and carrier solvent methods described above are representative of the natural toxicities of the NMs studied and whether methods to aid dispersal act synergistically or antagonistically on NMs causing their toxicity to be

misrepresented. In addition, methods of dispersion, such as sonication may themselves lead to increase toxicity through, for example, the generation of reactive oxygen species (ROS) (Handy et al., 2012a).

1.3. Aims, objectives and hypotheses:

The overall aim of this study is to examine the variation in life history response to a combination of a natural environmental variable (calcium availability) and a novel anthropogenic stressor (nanoparticle carbon black) in populations from across the UK geographic range of the great pond snail, *Lymnaea stagnalis*.

Main Hypotheses:

1.) Life history adaptation to prevailing environmental conditions may involve different trade-offs across the geographic range due to genetic and phenotypic variation.
2.) Adaptation to environmental conditions will influence the ability of populations to adapt to other stresses due to trade-offs in energy allocation.

Objectives:

1.) Analyse geographic variation in, and trade-offs between, life history traits of *L. stagnalis* across its UK range.
2.) Assess differences in the life history response of populations of *L. stagnalis* to a combination of low calcium availability and toxic substances, specifically nanoparticles.
3.) Link life history data to demographic models to determine the implications for population growth and the relative sensitivity of different life history traits.

1.4. Structure and aims of the thesis

Chapter 2 will utilise long-term (two generation) laboratory experiments at two levels of environmental calcium to address the following aims:

- Establish extent of variation of life history traits between populations and calcium treatments
- Distinguish between plastic and heritable components of variation in life history traits

Chapter 3 makes further use of the data from the long-term laboratory experiments to:

- Examine evidence for trade-offs between different traits and determine whether the pattern and extent of trade-offs varied between populations and calcium treatments
- Establish which life history traits contribute most strongly to the variation found.
- Parameterise a stage-classified matrix model and examine the sensitivity of population growth rates to variation in different life history components.

Chapter 4 focuses on toxicological exposures to nanoparticle carbon black and builds on the modelling framework developed in Chapter 3 to:

- Assess the effects of acute and chronic exposures to carbon black nanoparticles under varying environmental conditions (in the form of calcium availability)
- Utilise a stage-clasified matrix model to compare intra-specific responses at the population level, across three study poulations, to varying environmental calcium in conjunction with carbon black exposure.

Chapter 5 presents a final discussion of the main themes of the research undertaken.

2. Effects of environmental calcium on life history traits of *Lymnaea stagnalis*

2.1. Abstract

A long term study was conducted where nine different populations of the great pond snail, *Lymnaea stagnalis* collected from sites spread across the UK, were reared in high and low calcium environments for two generations in the laboratory. A comprehensive suite of life history traits were recorded to examine the extent of variation of life history traits between populations and treatments. Intergenerational comparisons of traits were then performed in an attempt to distinguish between plastic and heritable components of trait variation.

Significant intra-specific variation was recorded in the majority of life history traits measured with high levels of phenotypic plasticity being recorded throughout the study. Differences between traits were more readily detected at the population level rather than across calcium treatment, suggesting that either calcium has a less significant effect on *L. stagnalis*' life history than first proposed, or that calcium levels used in this study were not sufficient to elicit effects. High heritability, in the form of consistent patterns of variation across generations, tended to be detected in traits directly associated with fitness such as size at first reproduction, reproductive output and shell composition and shape. Traits pertaining to somatic growth, such as growth rates, age at first reproduction and shell weight to length ratios, tended to show a high degree of variability with respect to environmental calcium conditions, perhaps reflecting selection pressure imposed by environmental variability in this resource.

Observed life history trait variation appeared to present distinct, population specific life history strategies consistent with associated trade-offs between key life history components.

2.2. Introduction

Much of classical interpretation of variation in life history traits has focussed on optimality theory and bet hedging to explain patterns observed in principal life history traits such as size at birth, size and age at first reproduction, reproductive output, age specific mortality rates and lifespan (Stearns, 2000). Optimality theory aims to define a relationship between these principal traits and fitness, and attempts to explain a given life history strategy in terms of the combination of traits (and inherent trade-offs) that maximise fitness. Stearns (2000) highlights the limitations of the optimality approach, in that it assumes stable populations with constant mortality and fecundity (fitness) rates and that phenotypes are identical, representing a significant departure from reality. An alternative approach is 'bet-hedging', where fitness is assumed to vary over time and observed life histories are perceived to result from attempts to minimise the impacts of periods of low fitness rather than evolving to an optimum (Begon et al., 2006). A third approach considers that a given individual's life history is frequency dependent, relative to the other individuals within the population (Mappes et al., 2008), while a fourth aims to address the assumption of population stability by considering the observed life histories with respect to dynamic populations (Lande, 1982). All four approaches have their strengths and weaknesses, but despite its limitations optimality theory has been highly successful in explaining variation in major life history traits across and between genera and remains central to the understanding of life history evolution (Stearns, 2000, Begon et al., 2006, Parker and Smith, 1990).

Environmental conditions experienced by a species drive variation in different aspects of its life history (Paul-Pont et al., 2010, Balbontin et al., 2009, Morrison and Hero, 2003, Olsson and Agren, 2002). Given the importance of calcium to the development of freshwater molluscs (Russel-Hunter, 1964), and its known influence on aspects of ecology as diverse as distribution (Boycott, 1936, Briers, 2003), to memory formation (Dalesman et al., 2011), and in mediating responses to predator cues (Rundle et al., 2004), it may be expected that *L. stagnalis* may respond to variation in calcium availability through modification of life history traits. However, given the wide range of intra specific variation in life histories exhibited by populations of this and

similar species (Brown, 1979a, Brown, 1983), populations obtained from different geographical ranges may show marked differences in life history traits, depending on the environmental history of the sites sampled, the sensitivity of the traits to environmental variation and the range of variation (plasticity) inherently possible in the trait (Seigel and Ford, 2001, Trussell, 2000).

By rearing organisms in common-garden conditions for two generations or more it is possible to differentiate the extent of plastic responses from those derived from adaptive variation due to genetic factors (Ballentine and Greenberg, 2010). It is possible that selection acts not only at the level of specific life history traits but on the extent of trait plasticity in response to environmental variation (Stearns, 1989a, Scheiner, 1993). To assess the extent and heritable component of plasticity, the introduction of a variable environmental component such as temperature, pH or as in this study calcium availability, allows a measure of the level of phenotypic plasticity across environments and between generations to be measured

Here the aim is to utilise long-term (two generation) laboratory experiments at two levels of environmental calcium to establish patterns of inter-population variability, and the underlying phenotypic plasticity and heritability in the main life history traits of *L. stagnalis*.

2.3. Materials and methods

2.3.1. Field surveying

In order to determine life history variation across the species range, sampling of 9 populations throughout the UK was conducted between April and May 2007 (see Figure 2.1. and Figures 2.2.-2.9.). Site selection was based where possible on existing environmental data obtained from previous studies by Pond Conservation and Liverpool John Moores University. The data were used to select sites over a wide geographical spread that were relatively pristine and un-impacted by pollution as judged by environmental data, in order to minimise any impact that previous pollution may have had on life history traits. Populations were sampled from both the central and marginal areas of the range (see Figure 2.1.) from ponds and a single canal site, Glanahafren.

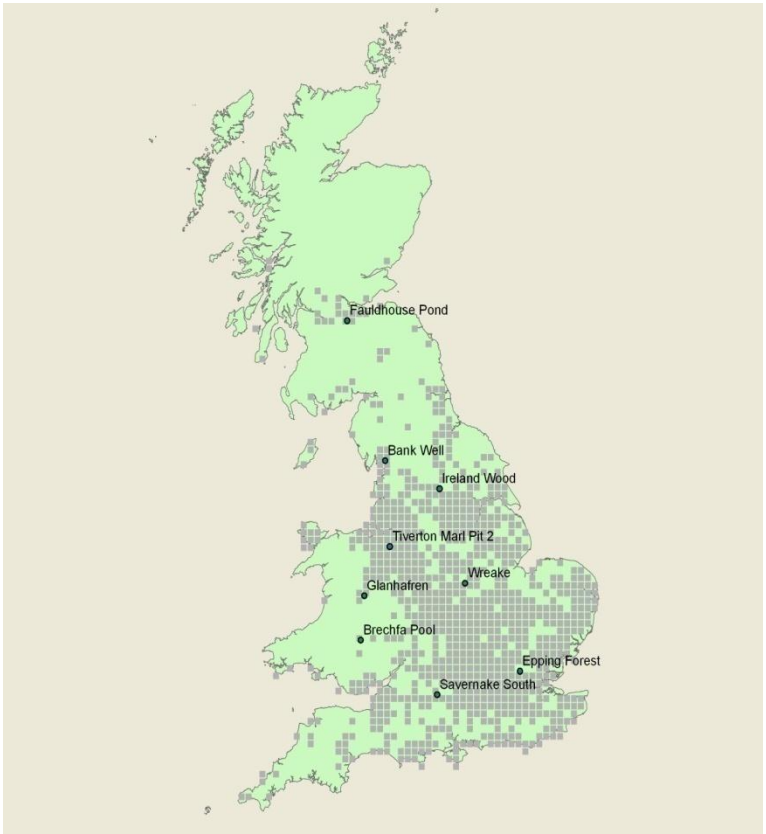


Figure 2.1. Sites sampled throughout the UK. Grey squares indicate 10km^2 tetrads where *L. stagnalis* has been recorded since 1970. (Country border data © Crown Copyright/database copyright 2012. An Ordnance Survey/EDINA supplied service. *L. stagnalis* distribution data based on records from the National Biodiversity Network.

Water samples from each site were taken in 500ml acid-washed PTFE bottles and frozen to allow nutrient analysis to be carried out at a later date. Nutrients in the form of Total Oxidised Nitrogen (TON), nitrite, Soluble Reactive Phosphorus (SRP), and ammonium were determined via SEAL AQ2+ auto analyser using USEPA equivalent methods.

The 9 sites sampled, and their three letter contractions used throughout in the text, tables and figures throughout this text are listed in Table 2.1:

Table 2.1. Site names, locations, and abbreviations used throughout the thesis.

Site name	Abbreviation	Location
Bank Well	**BAN**	**SD471754**
Brychfa	**BRY**	**S0119376**
Epping	**EPP**	**TQ416967**
Fauldhouse	**FHO**	**NS623910**
Glanahafren	**GLA**	**SO168964**
Ireland Wood	**IRE**	**SE256382**
Savernake	**SAV**	**SU221651**
Tiverton Marl Pit	**TIV**	**SJ535617**
Wreake	**WRE**	**SK623131**

Figure 2.2. Bank Well.

Figure 2.3. Epping Forrest.

Figure 2.4. Glanahafren, Montgomery canal.

Figure 2.5. Ireland Wood.

Figure 2.6. Savernake South.

Figure 2.7. Tiverton marl pit 2.

Figure 2.8. Wreake pond.

Figure 2.9. Fauldhouse pond. Displaying sampling method.

2.3.2. Calcium treatments

Artificial Pond Water (APW) medium (ASTM, 1980), modified to give two different calcium concentrations (see Table 2.2) was used for maintaining populations in the laboratory. Due to the tendency of the reagents to form a precipitate when preparing quantities for large volumes it was necessary to dissolve the calcium and potassium salts, and the magnesium and sodium salts separately and subsequently combine them. Experimental calcium concentrations were set to 40mgL^{-1} (low Calcium) and 200mgL^{-1} (high Calcium) to fall within the range of known values from sites across the geographic range (Pond Conservation: National Pond Survey data).

Table 2.2. Artificial Pond Water constituents for high and low calcium APW. Modified from ASTM (1980).

Ca^{2+} Conc. (mg L^{-1})	Salt concentration (mgL^{-1}, mmolL^{-1})			
	$CaCl_2.2H_2O$	$NaHCO_3$	$MgSO_4.7H_2O$	KCl
40	147, 1.00	64.5, 0.77	123.25, 0.5	5.75, 0.077
200	734, 4.99	64.5, 0.77	123.25, 0.5	5.75, 0.077

All individuals gathered from the field were held in separate tanks for each population initially in high calcium media as this was deemed to minimise stress in the stock populations and to promote reproduction.

2.3.3. Flow through apparatus: Description and maintenance

Flow through system.

A set of four flow through systems similar to that described by Van Der Steen *et al.* (1969) were constructed to maintain the populations under different calcium regimes. The apparatus comprised of four separate pumped systems each with eight identical tanks (thirty two in all) holding approximately 13L of water each. This resulted in two systems (A & C) being run at the low Ca concentrations and two (B & D) being run at the high Ca concentration.

Figure 2.10. Flow through apparatus. Displaying systems A-C, with lighting system clearly visible above tanks.

A photograph of the flow through apparatus is displayed in Figure 2.10 and a schematic of the apparatus is presented in Figure 2.11.

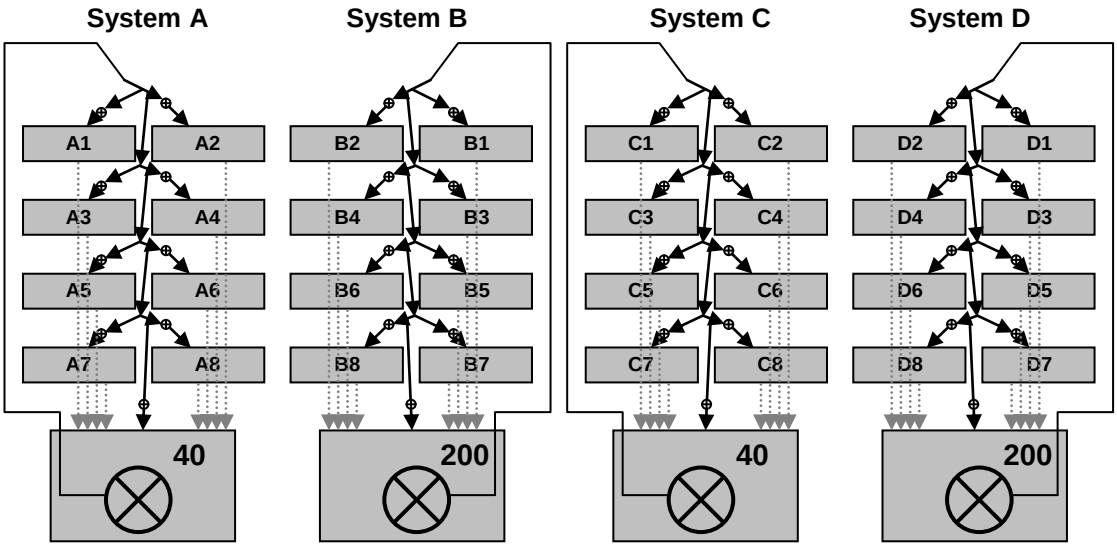


Figure 2.11. Schematic representation of flow through apparatus, displaying four individual pumped systems (A-D) and tanks 1-8 per system. Taps to regulate flow are represented by small crossed circles, while pumps are pumps located in sumps are represented by larger crossed circles. Calcium concentrations (mgL^{-1}) are indicated in sumps and direction of flow indicated by arrows (inflow = black and outflow = grey).

The flow through system was located in the controlled environment chamber at Edinburgh Napier University which was maintained at a constant temperature of $17^{O}C$ (see Figure 2.23) with a 12h: 12h, light: dark regime. The total volume of each system was approximately 165L. In order to account for water losses due to evaporation the systems were regularly (twice weekly) topped up to marks in the sumps with de-ionised water. Losses from evaporation were approximately 20L per system per week, with systems nearest the cooling extractor fan (beside system D) experiencing the greatest losses. Partial water changes were carried out monthly, where the sump was drained (after ensuring that the system had been topped to the mark with deionised water) and freshly prepared media replaced to the mark. The volume of water exchanged monthly was approximately 60L (greater than one-third of total system volume). APW media was made up in advance prior to changing and kept within the controlled environment facility to minimise any temperature shock to the organisms on water changes. Temperature in the constant temperature room and the tanks was monitored using a combination of LogIT datameter and Tinytag temperature loggers (both accurate to $0.1^{o}C$) depending on the availability of equipment.

2.3.4. Experimental design and procedure

F_1 Generation: Hatchling groups, isolated individuals, and adults:

A schematic of the experimental procedure and the project time line is displayed overleaf in Figure 2.12.

The basic experimental procedure began in July 2007 with egg masses being harvested from the nine field collected stock populations held externally in the high calcium (200mgL^{-1}) APW media. These egg masses were to serve as the foundation of the F_1 generation. Laying dates were recorded and the masses were evenly distributed in individual cups between the high or low calcium flow through systems in hatching cups (with gauze bases to allow water transfer – see Fig 2.13) and monitored until hatching occurred.

Figure 2.13. Cup used for egg masses and rearing of hatchlings.

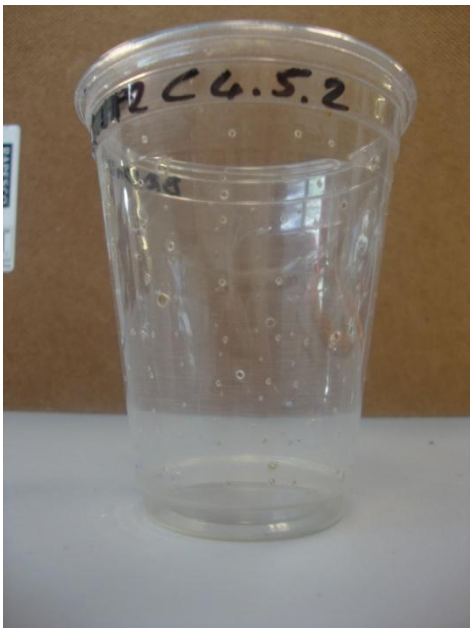


Figure 2.14. Cup used for isolated Individuals.

Upon hatching the date and number of individuals hatching was recorded and the animals were fed *ad libitum* with iceberg lettuce. Within four weeks of hatching the individuals were placed at constant densities of ten individuals per cup (hereafter referred to as *hatchling groups*). Six to seven replicates were established for each population and calcium concentration, with effort made to ensure that the individuals came from as diverse a selection of egg

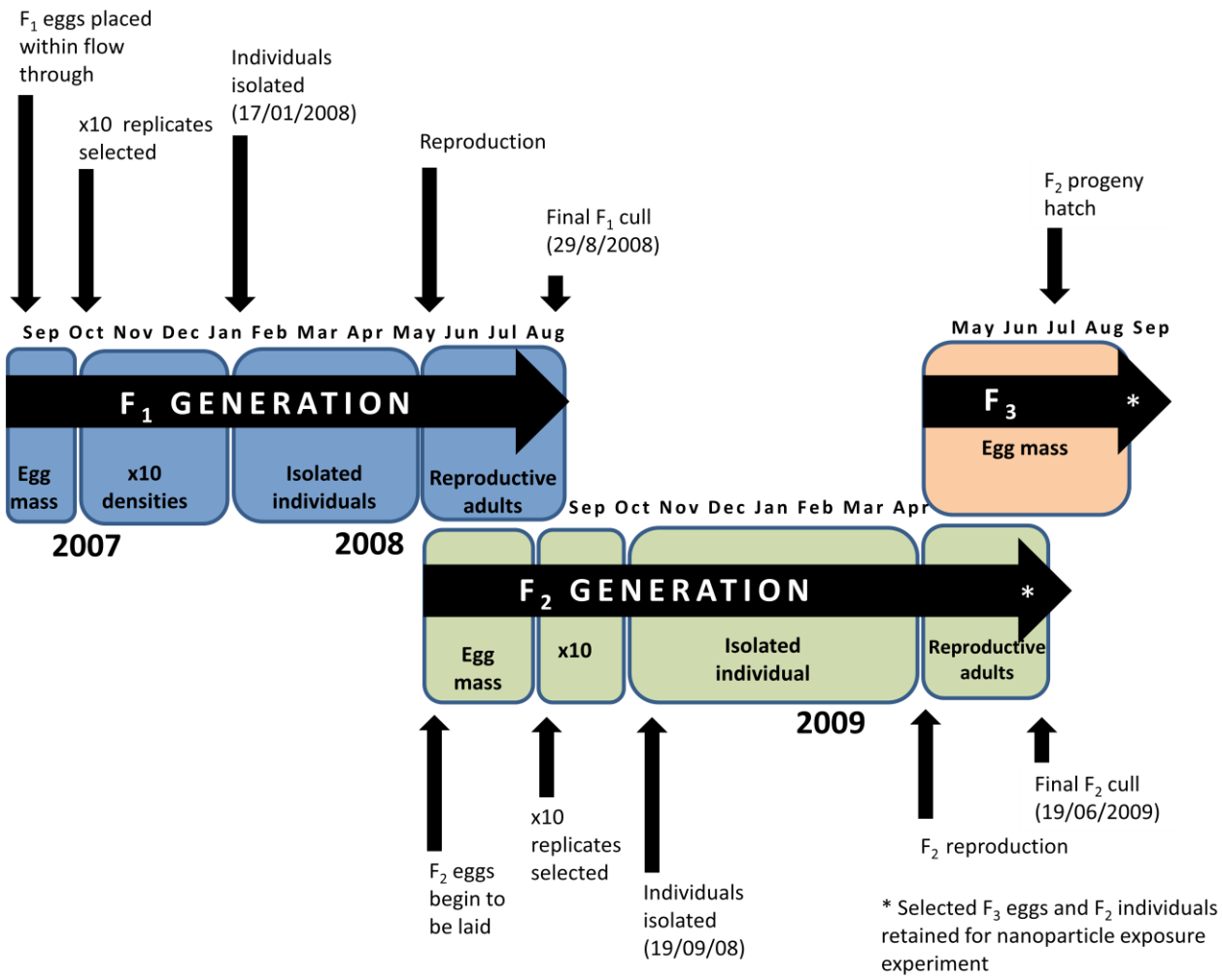


Figure 2.12. Schematic overview of procedures carried out within the flow through system between 2007 and 2009. Unless specific dates are given most temporal events are classified by the overall means of the whole sample group.

masses as possible. Populations were randomly distributed among the trays. Length measurements taken at fortnightly (via digital images) until the animals reached a size of approximately 10mm whereupon, on the 17th of January 2008, 20 individuals (hereafter referred to as *isolated individuals*) were isolated (in cups with small holes to allow water transfer – see Figure 2.14.) and their growth continued to be measured using digital images until such a point that it became possible to measure them with vernier callipers without risk of damage.

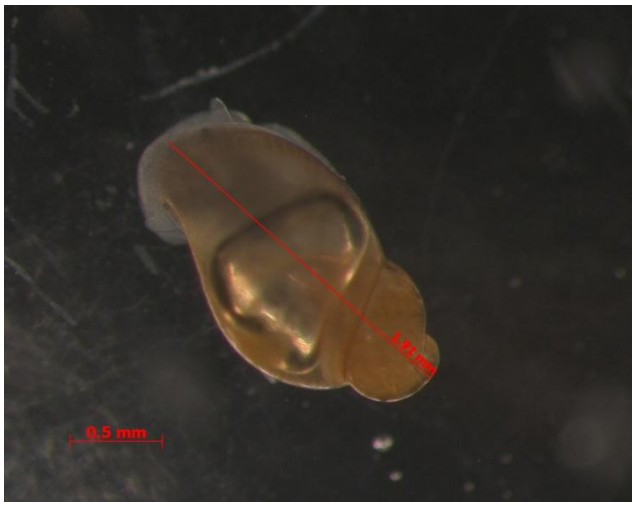


Figure 2.15. Length measurement on juvenile *L. stagnalis*.

Length measurement was defined as the distance from the anterior to the furthest point away on the spire of the shell (See Figure 2.15.). Again efforts were made to ensure that the isolated juveniles came from an even representation of the hatchling groups with typically no more than 4 juveniles being selected from the same egg mass. Isolated individuals' growth was recorded fortnightly (or monthly at later stages when growth was shown to slow) until either mortality or reproduction (by selfing) occurred, whereupon the individuals were classified as *adults*.

Upon deposition, egg masses were collected and photographed (to allow the number of embryos in each egg mass to be determined) before being placed in a hatchling cup (Figure 2.13) and returned to the appropriate calcium concentration within the flow through system. Reproductive output was then monitored for a minimum of 21 days after first recorded reproduction, whereupon the adult individuals were culled by being placed in boiling water. Adult shells were retained for subsequent analysis of shell calcium concentration. All non-reproductive individuals were culled on the 28th of August 2008.

F_2 generation:

Egg masses from F_1 adults were then allowed to hatch within the flow through system, with the date of hatching and the number of snails hatched being recorded for each mass. Hatchling success for each mass was given by the numbers of embryos within the ooetheca divided by the numbers hatched. As with the F_1 generation, 6-7 hatchling groups of 10 individuals were established and allowed to reach a size of approximately 10mm, whereupon on the 19th of September 2008, 20 juveniles from each treatment were isolated, with these individuals comprising the basis of the F_2 generation. In some cases, such as the F_2 Epping population at high calcium, due to high hatchling group mortality, it was not possible to get a full 20 individuals (n = 17) and those that were selected came predominantly from 2 egg masses.

Isolated individuals were reared until first reproduction, whereupon egg masses were collected as described for the F_1 generation and individuals were culled 21 days after 1st reproduction (or retained for further study – see Chapter 3). The remaining living non-reproductive individuals were culled on 19th of June 2009, bringing the F_2 stage and the main life history study to a close.

2.3.5. Environmental monitoring

Monitoring of flow through water quality

To assess temporal changes in water quality within the flow through system, samples were taken from the sumps and frozen prior to the monthly water changes being carried out. At a later date, water samples were assayed for TON, nitrite, ammonium and SRP via a SEAL AQ2 auto analyser.

2.3.6. Statistical analysis and modelling

System effects

Wherever possible, the potential effects of replicated being located in different systems of the flow-through were accounted for by including a 'block' effect in the analysis. However in no case was the block effect found to be significant, so the results reported in section 2.4 are from analyses without the block effect included.

2.3.6.1. Growth analysis and model selection

Hatchling groups

Growth rates in hatchling groups in the F_1 generation were determined by linear regression of average length of individuals in each replicate over time, allowing a growth rate (mm/day) for each replicate to be calculated. This process assumed that the replicates had entered a linear phase of growth (Zotin, 2009). ANOVA was then used to assess whether any differences in growth rates could be detected at the population or treatment levels.

Due to time constraints, only single length measurements for the F_2 hatchling groups were taken and as a result no analysis of initial growth rates could be performed. Instead sizes at the time of measuring were divided by mean age of individuals, to yield age-factored sizes. The data were normalised via log transformation and subjected to GLM ANOVA analysis.

Isolated individuals

Data derived from isolated individuals in both F_1 and F_2 data sets were analysed in the same manner, using R version 2.8 (R Core Development Team, 2009). Individual growth curves for all individuals were plotted using the lattice package. Any individuals which had a low number (<5) measurements and any which displayed little growth (non-viable) were removed from the data set. The non-linear mixed effects (nlme) package was then used to fit the resultant data set to four non-linear growth functions (Gompertz, Weibull, 3-Parameter Logistic and 4-parameter Logistic).

The four non-linear growth functions fitted to the data are described in equations 2.1. to 2.4.

$$y = a - be^{-(cx^d)} \quad (2.1.)$$

Equation 2.1. Weibull growth model.

$$y = a + \frac{b-a}{1+e^{(c-x)/d}} \quad (2.2.)$$

Equation 2.1. 4-parameter logistic growth model.

$$y = \frac{a}{1+be^{-cx}} \quad (2.3.)$$

Equation 2.3. 3-parameter logistic growth model.

$$y = ae^{-be^{-cx}} \quad (2.4.)$$

Equation 2.4. Gompertz growth model.

When assessing alternative models, there are two general criteria to be applied; firstly does the fit of the model make biological sense and secondly how well does the model fit to the observed data (Anderson and Burnham 2002). In order to assess the fit of the models to the observed data, Akaike Information Criterion (AIC) values were calculated for each individual model fit. AIC analysis is effectively a non-parametric measure of goodness of fit

which is valid for non-linear regression models (Crawley, 2007). The lower the value of the AIC the better the model fits the data.

Both the F_1 and F_2 AIC values were found to be normally distributed and a one-way ANOVA performed for each generation to determine whether any difference in fit existed between the four models. The ANOVA output alongside the mean AIC values for each model are displayed in Table 2.3.

Table 2.3. Summary ANOVA table to compare AIC values across fitted models for F_1 and F_2 generations.

Model	*Mean AIC*	*S.D.*	*D.F*	*F Statistic*	P-value
Gompertz F_1	23.57	9.63	3, 1137	5.67	0.001
3-P.L. F_1	23.46	10.16			
4-P.L. F_1	20.90	11.22			
Weibull F_1	21.02	10.25			
Gompertz F_2	21.65	8.66	3, 1407	60.01	<0.001
3-P.L. F_2	19.93	8.44			
4-P.L. F_2	15.08	8.51			
Weibull F_2	14.52	8.74			

Both the F_1 and F_2 data sets displayed significant differences in AIC values with the 4-parameter logistic and the Weibull models having significantly lower values than the Gompertz and the three parameter models in both instances (post hoc Tukey test, $P < 0.05$). This suggested that the Weibull and 4 parameter logistic models should be chosen as they displayed the lowest mean AIC values in each case.

However, the second criterion employed was the biological realism that was inherent in the fitted curves. Both the Weibull and the 4-parameter logistic models were assessed and considered to be poor descriptors of growth as the y-intercept values (at time=0) were found to display biologically inappropriate values (high positive or negative initial lengths). These models were therefore removed from further consideration. This resulted in the selection of the Gompertz growth model, which despite displaying the largest AIC values, was deemed to provide the most biologically appropriate fit to the dataset. Although the AIC values for this model were the highest on average when comparing the different models, the low deviance between minimum

and maximum values indicates that all models considered have substantial support on the basis of this criterion (Anderson and Burnham, 2002).

The Gompertz model is presented by Equation 2.4. The parameter terms *a*, *b*, and *c* are defined as follows:

> ***a*:** a numeric parameter representing the horizontal asymptote on the right side (maximal growth);
> ***b*:** a numeric parameter relating to the function at x=0;
> ***c:*** a numeric parameter relating to the scale of the x axis reflecting growth rate.

Values of each of the three parameters *(a, b* and *c*) of the fitted Gompertz model for each individual were then used to determine if particular growth parameters varied between populations and treatment using ANOVA. Some of the parameter terms were not normal and appropriate transformations were applied to normalise the data (natural log, inverse reciprocal or square root).

2.3.6.2. Survivorship

Hatchling groups

Mortality for the F_1 generation hatchling groups was analysed using non-parametric Kaplan-Meier survivorship analysis (right censored). Censoring occurred when individuals were removed from replicates for subsequent isolation (see below). Survivorship data for the F_2 generation hatchling groups were only measured as proportional survival at the end of the x10 period and were thus analysed using ANOVA. Here mortality data from each hatchling group replicate were converted into percentages and Kruskall-Wallis tests performed to determine whether any differences between mortality could be found at the population level and in response to calcium treatment.

Isolated individuals

Mortality in the isolated individuals was analysed using non-parametric Kaplan-Meier survivorship analysis with right-censoring. Censoring was carried out for any individuals that were culled or killed accidentally by

drowning which could occur when cups were not righted and became completely immersed.

2.3.6.3. Reproduction

Adult age at first reproduction was square root transformed to yield normal data and analysed via ANCOVA, with age at isolation as a covariate. Size at first reproduction was determined via interpolation of each individual's Gompertz derived growth curve for their specific age at first reproduction. Shell length at first reproduction was then analysed via ANCOVA, with age at isolation as a covariate (where significant).

Reproductive output was analysed both in terms of the number of eggs and the number of egg masses per reproducing individual in the 21 day period after first reproduction. The number of eggs per mass was also analysed. If required, analyses used appropriate data transformations (square root) to normalise the data and allow ANOVA to be performed to assess whether differences in reproductive output existed between population and treatment.

Egg survivorship was defined by dividing the number of embryos per mass over the number of individuals hatched. ANOVA was then used to assess whether any difference in egg survivorship could be found between populations and calcium treatments.

2.3.6.4. Shell weight and calcium content

Shells from all culled reproductive individuals (F_1 and F_2) were dried in an oven at 50^{o}C for 12h and weighed. Analysis of the dry shell weight data by ANCOVA was performed after log transformation. Age at cull and shell length were used as covariates.

The shells from three randomly selected F_1 generation individuals from each population and calcium treatment, and all of those from the Fauldhouse population, were then subjected to acid digestion to determine calcium content of the shells. Snail shells of known weight were dissolved in 10ml of

10% HNO_3 (Aristar grade) and evaporated via a hotplate to a volume of approximately 1ml. Evaporated samples were then filtered through 2.5cm^2 GFC filter papers into a 25ml volumetric flask and made up to the 25ml mark with 2% HNO_3 (Aristar grade). Blanks were prepared by evaporating 10ml HNO_3 and filtering in the same manner. Standards were prepared from Fisher brand 1000ppm calcium solution diluted stepwise to 100, 10 and 1ppm in 2% HNO_3 (Aristar grade).

Shell calcium content was analysed using Inductively Coupled Plasma – Atomic Emission Spectroscopy (ICP-AES) at the University of Edinburgh with the 315.887nm absorbance line being used as this gave the best standard curve ($R^2 > 0.9999$).

Statistical analysis was then performed to determine the relative calcium content of the shells to be compared across population and treatment. This involved natural log transformation of the data which were subsequently analysed using ANCOVA, with dry weight as a covariate.

2.3.6.5. Shell morphology

Shell morphometric measurements from the F_1 and F_2 generations were measured using a Zeiss Cam MRc microscope. Snails were measured in a consistent manner, with the operculum facing upwards and the shell supported in a cupped dish to best maintain the shell's orientation relative to the plane of the camera. The morphological features measured and the abbreviations used throughout the rest of the text are displayed in Figure 2.16.

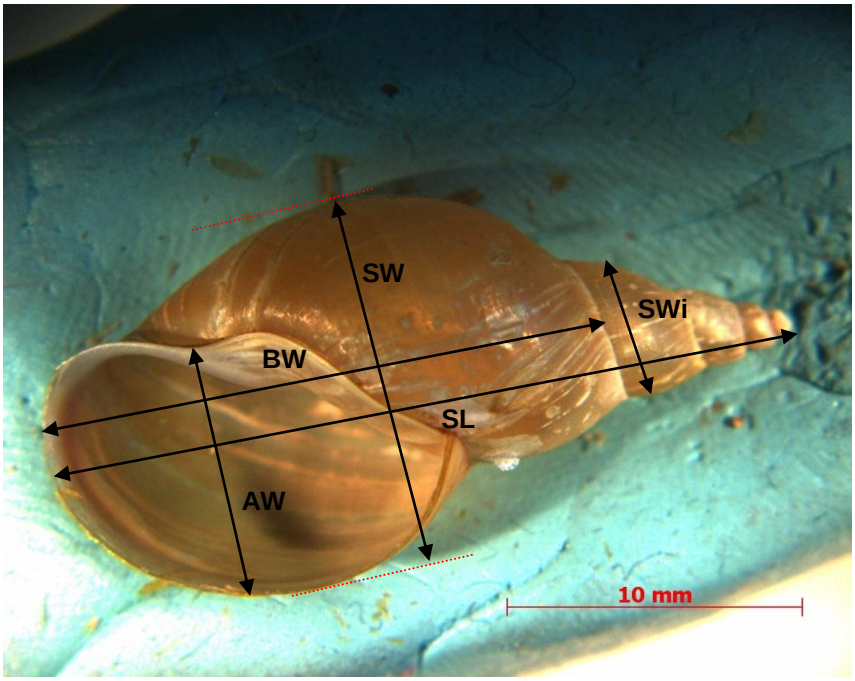


Figure 2.16. Shell morphology measurements : SL = Shell length, SW = Shell width, AW = Aperture width, BW = Body whorl length, SWi = Spire width.

Due to the fact that three of the F_2 populations were used in a further experiment (see Chapter 4) it was only possible to measure morphological characteristics for six of the F_2 populations. Morphological data were analysed using Multivariate Analysis of Variance (MANOVA) and Principal Component Analysis (PCA). Analysis of individual components was then carried out by ANOVA with population and calcium level as main effects.

2.3.6.6. Inter-generational comparisons

All measured life history traits from both the F_1 and F_2 generations: growth parameters (*a*, *b*, *c*), age at first reproduction, reproductive output (number of eggs and number of egg masses in 21 days, number of eggs per mass), shell weight and survivorship were compared to assess whether findings remained consistent across generations. The process involved plotting the mean F_1 and F_2 values of a particular trait and performing linear correlations with the resultant Pearson's product-moment coefficient (Zar, 1999) giving the degree of linear dependence between trait values across generations.

2.4. Results

2.4.1. Environmental monitoring

Site nutrient data: Calcium

The results of ICP determined site calcium data are displayed in Figure 2.17. Calcium was found to be lowest in the Savernake and Fauldhouse sites and highest at the Wreake and Ireland wood sites with the remaining sites (Tiverton Marl 2, Glanahafren, Brychfa, Bank Well and Epping) displaying intermediate levels. Environmental calcium was shown to range between 2.11mgL^{-1} at the Savernake site to 80.6mgL^{-1} at the Ireland Wood site.

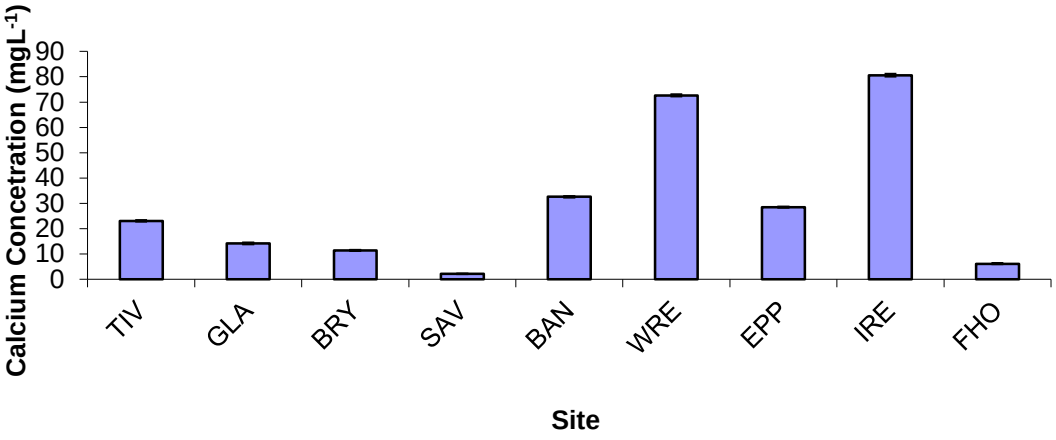


Figure 2.17. Site calcium concentrations. Error bars denote standard deviation.

Nitrate

Site nitrate concentrations are displayed in Figure 2.18. In general nitrate concentrations were found to be low at all sites, however higher concentrations were found at Wreake, Glanahafren and Brychfa sites, with the highest nitrate value of 2.63 mgL^{-1} being recorded at the Wreake site.

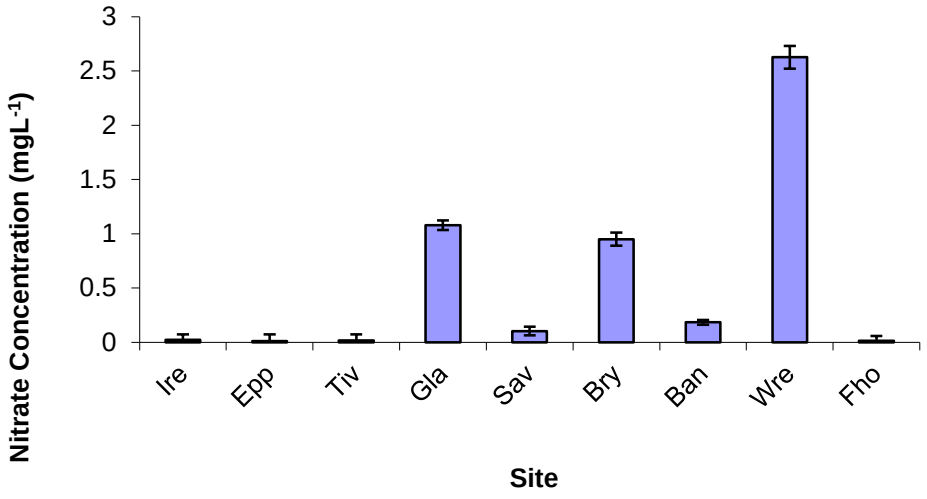


Figure 2.18. Site nitrate concentrations. Error bars denote standard deviation.

Nitrite

Site nitrite concentrations are displayed in Figure 2.19. Nitrite concentrations ranged from 0.0216mgL^{-1} at Epping to the highest values of 0.264mgL^{-1} recorded at the Bank Well site. The Bank Well site is shown to display nitrite values at least double those of the other sites.

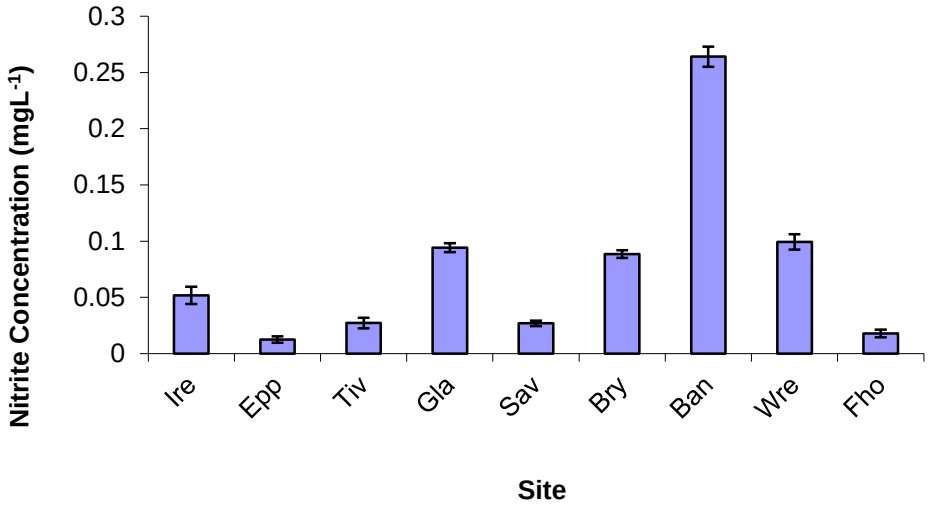


Figure 2.19. Site nitrite concentration. Error bars denote standard deviation.

Total Oxidised Nitrogen

Total Oxidised Nitrogen (TON) concentrations are displayed in Figure 2.20. TON concentrations ranged from 0.0241mgL^{-1} at Epping to the highest values of 2.73mgL^{-1} recorded at the Wreake site. The Wreake, Glanahafren and

Brychfa sites all displayed TON values greater than the other sites, with the majority of the TON being derived from nitrate content (see Figures 2.18 and 2.19).

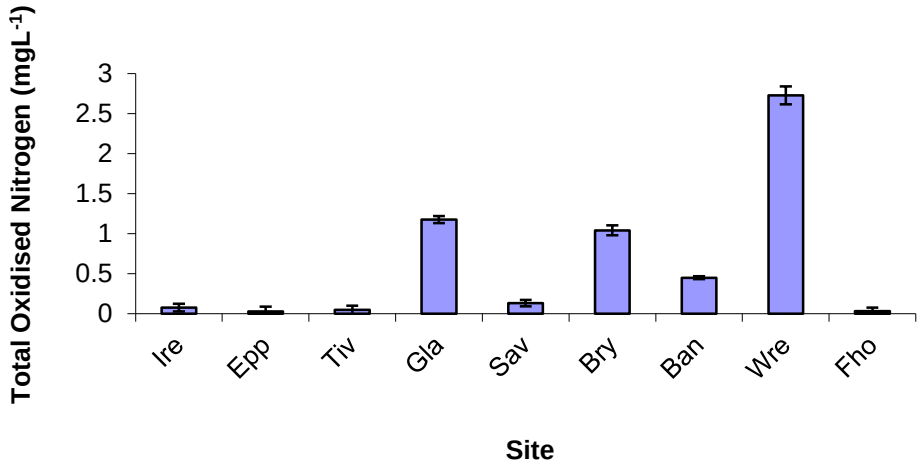


Figure 2.20. Site TON concentration. Error bars denote standard deviation

Ammonium

Site ammonium concentrations are displayed in Figure 2.21. Ammonium concentrations were found to be greatest in the Bank and Ireland sites. Ammonium concentrations ranged between 0.00211mgL^{-1} at the Fauldhouse site to 0.906mgL^{-1} at the Ireland Wood site.

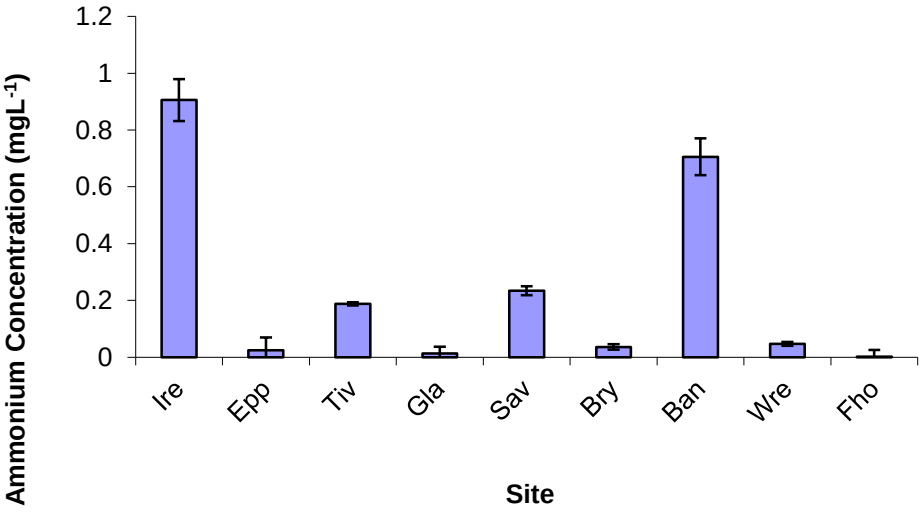


Figure 2.21. Site ammonium concentration. Error bars denote standard deviation.

Phosphate

Site phosphate concentrations are displayed in Figure 2.22. Savernake and Bank Well sites displayed the highest phosphate concentrations. Site phosphate concentration ranged from 0.0051mgL^{-1} in the Epping site to 0.0825mgL^{-1} in the Savernake site.

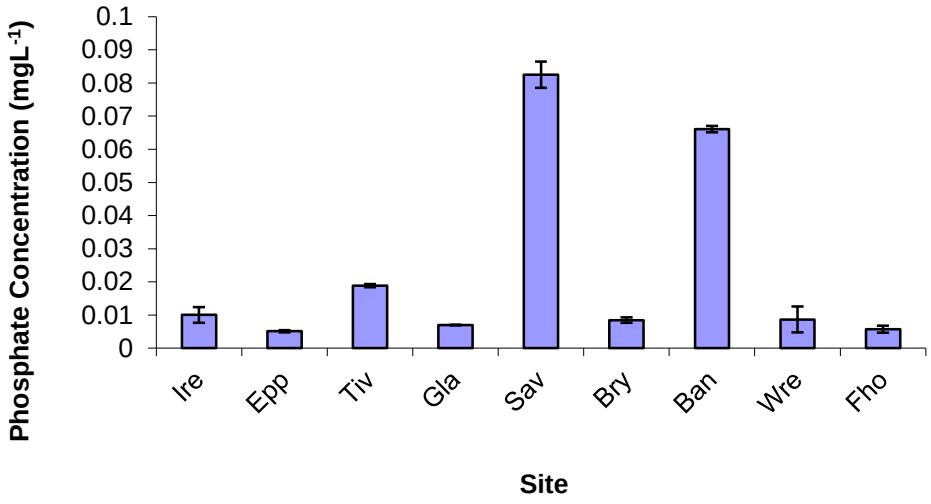


Figure 2.22. Site phosphate concentration. Error bars denote standard deviation.

Temperature

The data logger output displayed in Figure 2.23 shows the typical daily temperature variations within the system and the air temperature of the growth room. It can be seen in Figure 2.23 that diurnal temperature variation of approximately one degree takes place. This is most likely attributable to the heating effects of the lighting system.

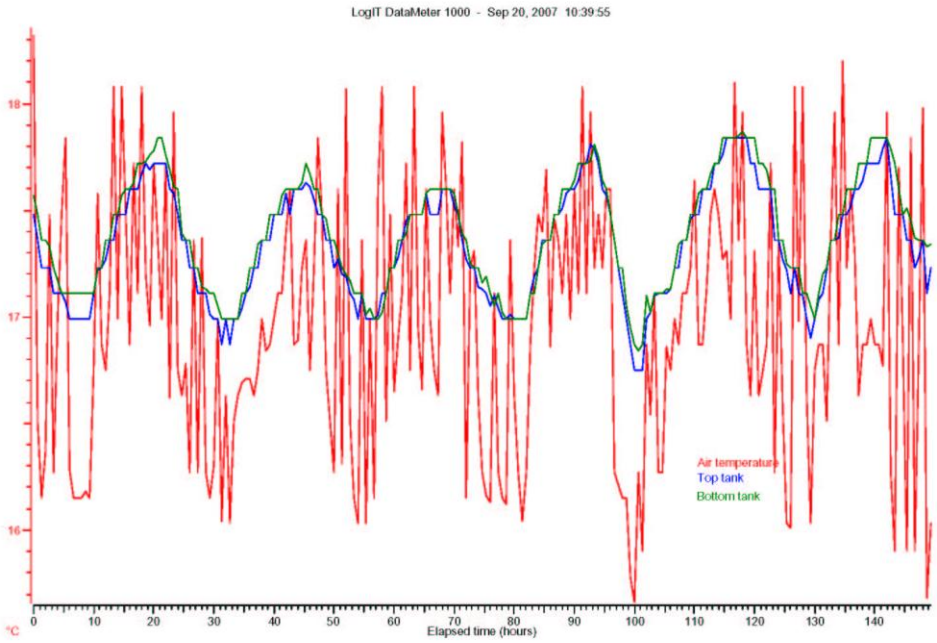


Figure 2.23. Example of daily temperature variation within the flow through systems and between top (blue) and bottom (green) tanks. Data from a six day period starting on September 20th 2007. Daily temperature fluctuations (±0.5°C) are most likely caused by the timed lighting systems.

Figures 2.24 and 2.25 display recorded long term variation in daily water and air temperatures within the growth room. Figure 2.24 displays sample data derived from June-September 2008, while figure 2.25 displays data from March-July 2009. It can be seen in figures 2.24 and 2.25 that some diurnal temperature variation takes place. This is most likely attributable to the heating effects of the lighting system. It would appear that the cooling system failed on or around the 15/06/08 (see Figure 2.24) as can be seen by the spike in the water temperature. It is possible the system reset may have been accidentally triggered at this time and subsequently caused the observed increase in water temperature. No mortality was recorded over this period and it is not likely that it would have a significant effect on any results obtained.

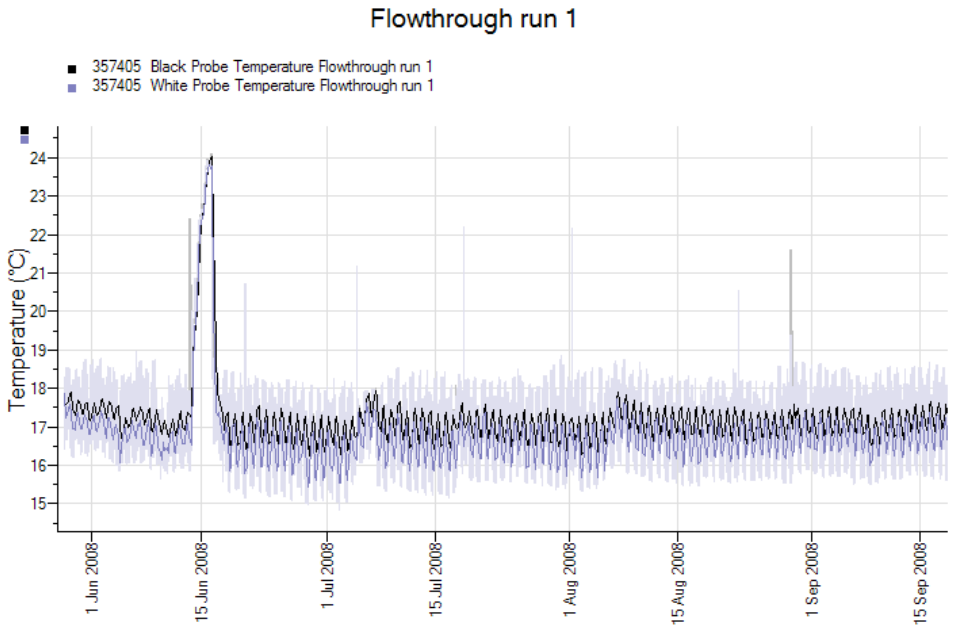


Figure 2.24. Air and water temperature values (blue and black respectively) in the flow through system between June and September 2008. Daily temperature fluctuations are most likely caused by the timed lighting systems.

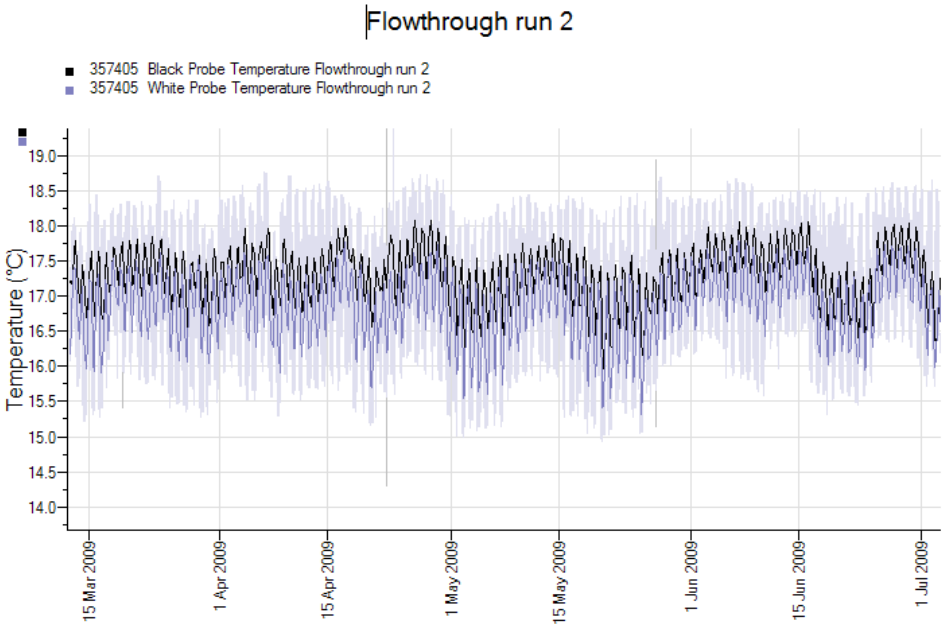


Figure 2.25. Air and water temperature values (blue and black respectively) in the flow through system between March and June 2009. Daily temperature fluctuations are most likely caused by the timed lighting systems.

From Figures 2.24 and 2.25 it can be deduced that the growth room maintained a stable temperature of 17 ±1^{o}C for the duration of the experiment.

The conductivity, dissolved (and % saturation) oxygen and pH values for each system (taken at the start of the experiments on 24/09/07) are displayed in Table 2.4. Calcium values determined by ICP-AES for samples taken on 19/06/2009 are also displayed in Table 2.4.

Table 2.4. Conductivity, O_2 (dissolved & % saturation) and pH values for systems A-D taken on 24/09/07. Calcium concentrations taken on 19/06/09 with standard deviation.

Measurement	**SYSTEM**			
	A	**B**	**C**	**D**
Dissolved O_2 (mgL^{-1})	9.70	9.78	9.74	9.71
% O_2 Saturation	100.2	100.6	98.5	98.1
pH	6.3	6.3	6.7	6.6
Conductivity (μscm^{-1})	324	1210	330	1177
ICP Calcium (mgL^{-1})	33.11 ±0.0636	196.52 ±0.524	39.66 ±0.253	185.44 ±1.61

It is of interest to note the difference in conductivity between the low calcium systems (A & C) and the high calcium systems (B & D) as a result of the higher concentration of calcium chloride dihydrate salts.

Flow-through nutrient data: Nitrogen

Total Oxidised Nitrogen (TON) values for systems A-D for the study period (2007-2009) are displayed in Figure 2.26. TON levels are shown to rise in all systems from the beginning of the experiment in 2007, before reaching a peak at around 4mgL^{-1} after approximately a year. There is inconsistent variation between the different systems, although all systems generally exceed the range shown by the sites where organisms were obtained.

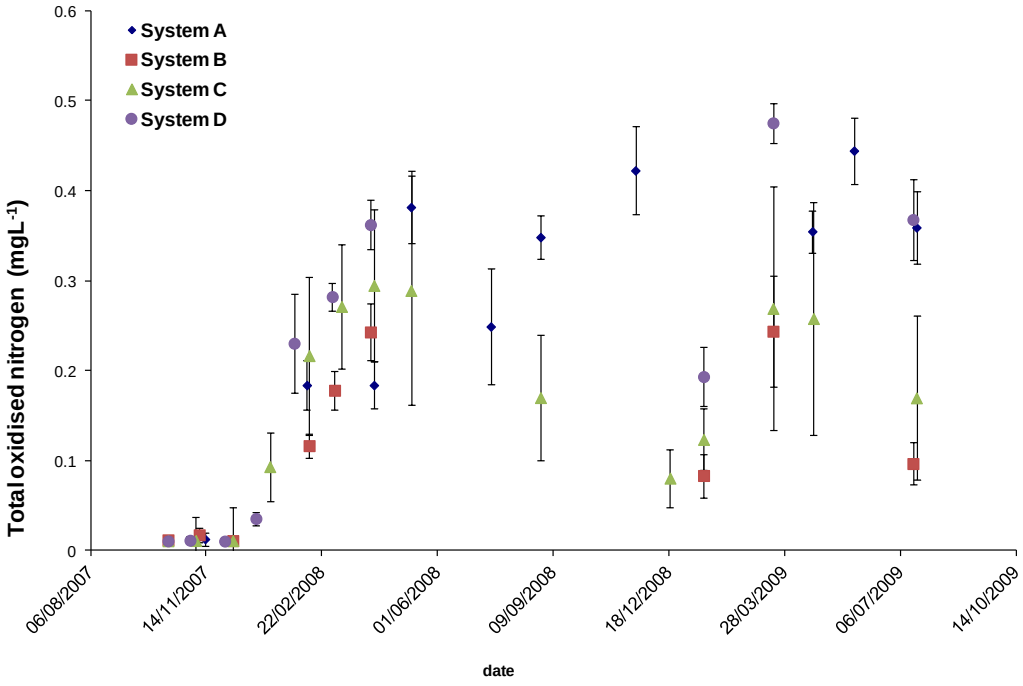


Figure 2.26. Total Oxidised Nitrogen (combination of nitrate and nitrite) values, systems A-D, 2007 – 2009. Error bars show standard deviation.

Ammonium

Ammonium values for systems A-D throughout the study period are displayed in Figure 2.27. Ammonium values were shown to rise steadily from 0.1mgL^{-1} to a peak of around 0.15 to 0.3mgL^{-1} by early 2008, after which ammonium concentrations fell to about 0.05mgL^{-1} in all systems for the remainder of the study period.

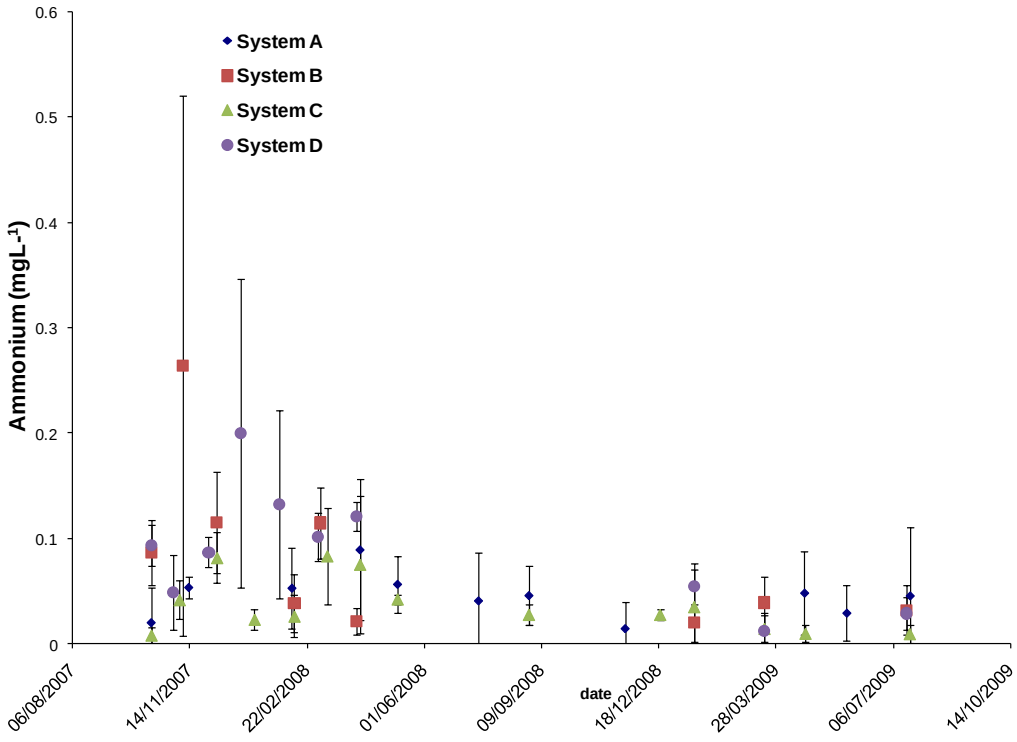


Figure 2.27. Ammonium values, systems A-D, 2007 – 2009. Error bars show standard deviation.

Phosphate

Phosphate values for systems A-D throughout the study period are displayed in Figure 2.28. Phosphate values rose steadily throughout the experiment to reach values of 1mgL-1 by the end of the study. It is worth drawing attention to the fact that the phosphate levels in system B do not rise accordingly with the other systems. Algal contamination was noted in all systems throughout the study, with the densest algal growth being observed in system B. It is therefore likely that the observed reduction in phosphate in system B is attributable to uptake by the algae.

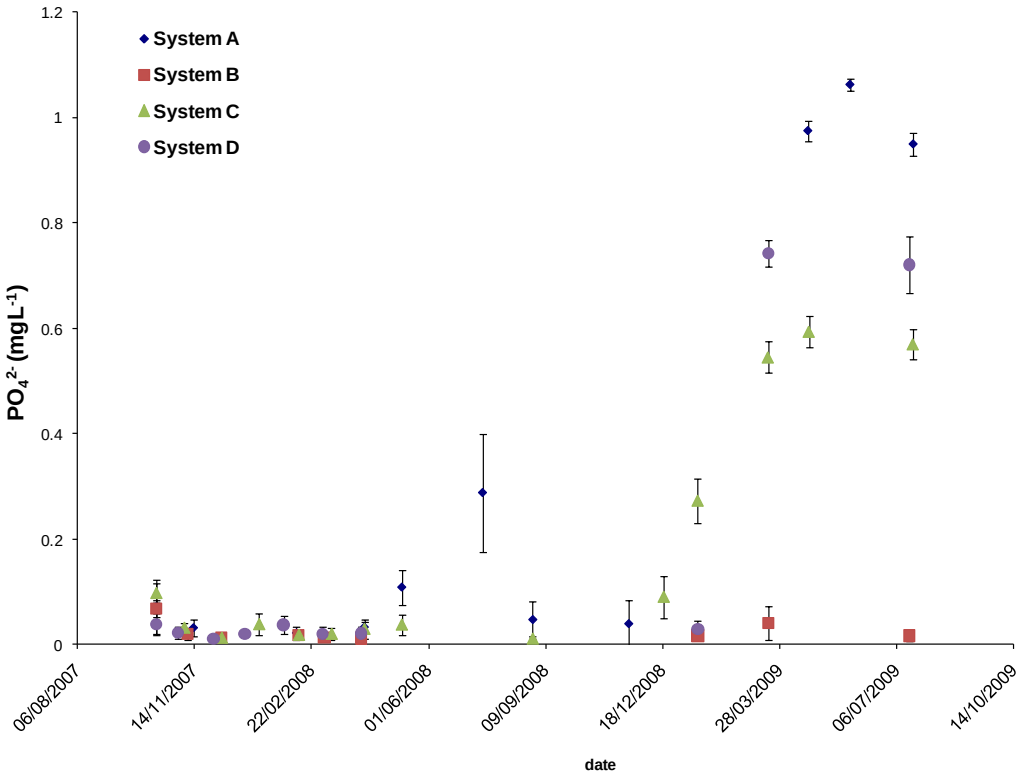


Figure 2.28. Phosphate values, systems A-D, 2007 – 2009. Error bars show standard deviation.

2.4.2. Growth

2.4.2.1. F_1 growth

Hatchling groups

Growth rate data from the F_1 hatchling groups were found to be normally distributed and were analysed using ANOVA (see Table 2.5).

Table 2.5. Summary ANOVA table for F_1 hatchling group growth.

Source	*D.F*	*F Statistic*	*P-value*	Significant
Population	8, 98	3.89	0.001	Y
Calcium	1, 98	2.56	0.113	N
Population * Calcium	8, 98	7.72	0.010	Y

There was a significant difference in early growth at the population level and a significant interaction between calcium treatment and population, suggesting that populations varied in their response to calcium treatment. However post-hoc Tukey tests did not detect significant differences within populations in response to calcium levels. Although the mean initial growth rates displayed in Figure 2.29 are suggestive of some differences, these are clearly not strong.

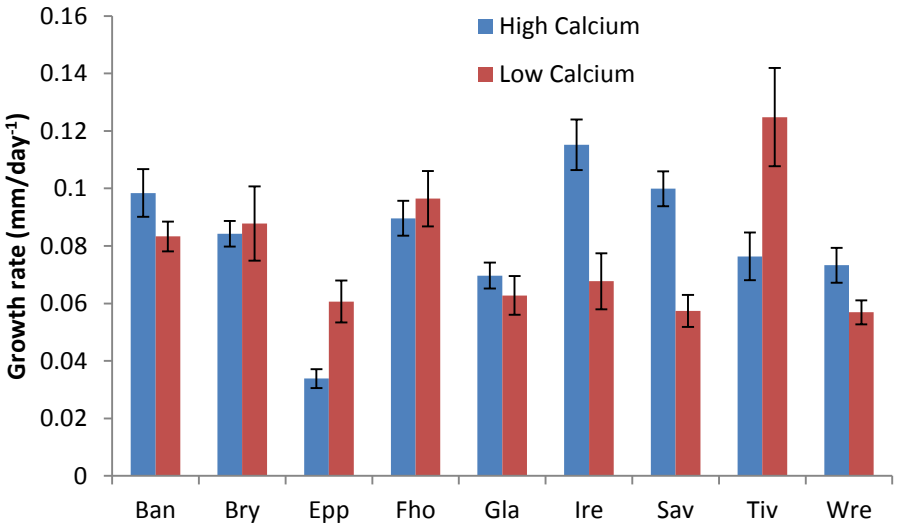


Figure 2.29. Mean hatchling group growth rates for F_1 individuals from different populations at high and low calcium treatment. Error bars denote standard error of means.

Isolated individuals

Table 2.6 displays the mean parameter terms of the Gompertz growth model (*a*, *b*, and *c*) for each population with 95% confidence limits. The derived growth curves for each population (at high and low calcium treatment) are displayed in appendices 6.1 to 6.9.

Table 2.6. F_1 Gompertz parameter values with confidence limits for F_1 isolated individuals.

Population	***Calcium***	**Mean parameter term**		
		***a* (± 95% C.I.)**	***b* (± 95% C.I.)**	***c* (± 95% C.I.)**
Bank	Hi	28.43 (25.43, 32.21)	20.23 (9.17, 34.77)	0.981 (0.976, 0.983)
	Lo	29.75 (27.56, 32.40)	11.15 (8.25, 14.62)	0.980 (0.978, 0.983)
Brychfa	Hi	34.47 (30.36, 39.15)	5.23 (3.28, 8.16)	0.986 (0.982, 0.989)
	Lo	30.84 (28.56, 33.39)	30.24 (13.50, 50.26)	0.978 (0.975, 0.982)
Epping	Hi	31.39 (29.69. 33.19)	16.22 (11.33, 21.10)	0.983 (0.980. 0.985)
	Lo	31.22 (28.95, 34.29)	11.38 (8.59,14.44)	0.983 (0.981, 0.985)
Fauldhouse	Hi	33.49 (29.08, 42.07)	11.98 (4.36, 23.09)	0.980 (0.974, 0.984)
	Lo	32.70 (31.60, 33.73)	12.06 (9.93, 15.22)	0.980 (0.978, 0.981)
Glanahafren	Hi	31.54 (29.56, 33.35)	10.58 (8.10, 13.39)	0.984 (0.982, 0.986)
	Lo	30.76 (29.71, 31.88)	11.41 (7,82, 15.60)	0.983 (0.980. 0.985)
Ireland	Hi	29.55 (26.27, 33.22)	5.05 (3.81, 6.61)	0.984 (0.980. 0.987)
	Lo	27.84 (26.16, 29.97)	55.17 (18.48, 106.7)	0.975 (0.972, 0.980)
Savernake	Hi	31.82 (29.23, 33.20)	8.27 (4.57, 12.84)	0.984 (0.980, 0.986)
	Lo	29.70 (27.65, 31.63)	33.84 (15.41, 51.65)	0.979 (0.974, 0.982)
Tiv	Hi	26.72 (24.54, 28.58)	7.21 (4.28, 11.37)	0.980 (0.977, 0.983)
	Lo	29.99 (27.77, 31.24)	4.37 (3.52, 5.29)	0.984 (0.982, 0.987)
Wreake	Hi	33.33 (31.69, 34.79)	10.78 (8.28, 13.93)	0.984 (0.983, 0.986)
	Lo	29.34 (27.79, 31.18)	26.95 (8.28, 13.93)	0.977 (0.983, 0.986)

Analysis of F_1 growth curves

In order to examine growth in more detail each individual parameter of the Gompertz model was analysed independently.

Parameter a

Parameter *a* represents the asymptotic shell length as time tends towards infinity. Thus parameter *a* can be used to glean insight into overall size differences across populations.

The inverse reciprocal of parameter *a* was taken to normalise the data and ANOVA performed. The results of the ANOVA are summarised in Table 2.7 and the mean parameter *a* values are displayed in Figure 2.30. Again, despite the significant interaction, post-hoc tests did not detect any significant differences in response to calcium within populations.

Table 2.7. Summary ANOVA table for F_1 parameter term *a* (inverse reciprocal).

Source	*D.F*	*F Statistic*	*P-value*	Significant
Population	8, 302	3.17	0.005	Y
Calcium	1, 302	0.28	0.599	N
Population * Calcium	8, 302	2.45	0.014	Y

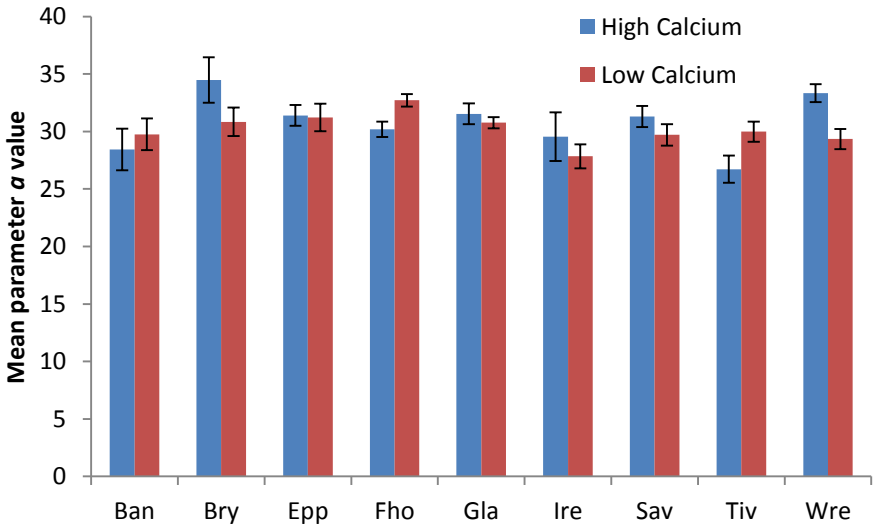


Figure 2.30. Mean parameter *a* values for different populations and calcium treatments. No significant differences between high and low calcium treatment were found in any population (post- hoc Tukey test, P <0.05). Error bars display standard error of mean.

Parameter b

Parameter *b* represents the initial shell length at t = 0. Parameter *b* was natural log transformed before being analysed by ANOVA. The results of the

analysis are displayed below in Table 2.8 while Figure 2.31 displays the raw, mean values of parameter *b*.

Table 2.8. Summary ANOVA table for F_1 parameter term *b* (natural log transformed).

Source	*D.F*	*F Statistic*	*P-value*	Significant
Population	8, 302	2.83	0.005	Y
Calcium	1, 302	21.12	<0.001	Y
Population * Calcium	8, 302	6.54	<0.001	Y

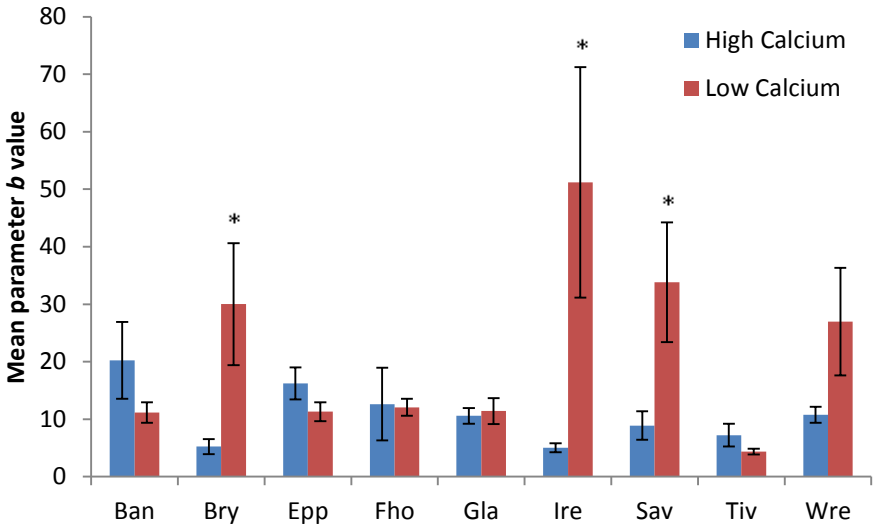


Figure 2.31. Mean parameter *b* values for different populations and calcium treatments. Significant differences between high and low calcium treatment at the population level are indicated by asterisks (post-hoc Tukey test, P <0.05). Error bars display standard error of mean.

Significant differences in parameter *b* values between high and low calcium treatment were found in the Brychfa, Ireland and Savernake populations (post hoc Tukey test, p<0.05).

Parameter c

Parameter *c* reflects growth rate and values were subjected to natural log transformation before being analysed by ANOVA. The results of the analysis are displayed below in Table 2.9 while Figure 2.32 displays the raw, mean values of parameter *c*.

Table 2.9. Summary ANOVA table for F_1 parameter term c (natural log transformed).

Source	*D.F*	*F Statistic*	*P-value*	Significant
Population	8, 302	1.85	0.067	N
Calcium	1, 302	9.90	0.002	Y
Population * Calcium	8, 302	3.36	0.001	Y

Intra-population differences between calcium treatment were only found in the Brychfa population (post hoc Tukey test, p <0.05).

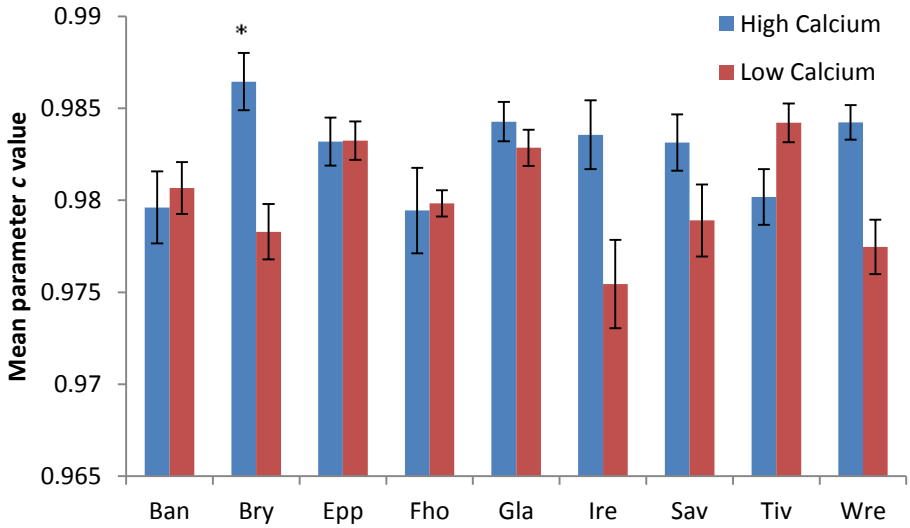


Figure 2.32. Mean parameter *c* values for different populations and calcium treatments. Significant differences between high and low calcium treatment at the population level are indicated by asterisks (post-hoc Tukey test, P <0.05). Error bars display standard error of mean.

2.4.2.2. F_2 growth

Hatchling groups

Age-factored sizes were normalised via log transformation and subjected to ANOVA analysis. Population was found to have a significant effect on length/age ratio, as was calcium and a significant interaction between population and calcium was also shown to exist (see Table 2.10).

Table 2.10. Summary ANOVA table for F_2 hatchling groups age-factored length (natural log transformed).

Source	*D.F*	*F Statistic*	*P-value*	Significant
Population	8, 393	4.41	<0.001	Y
Calcium	1, 393	15.30	<0.001	Y
Population * Calcium	8, 393	4.16	<0.001	Y

Post-hoc comparisons revealed that only the Fauldhouse population displayed a significant difference in length between calcium treatments (Tukey test, P<0.05). Figure 2.33 displays the raw mean length/age ratio values for each population/calcium treatment.

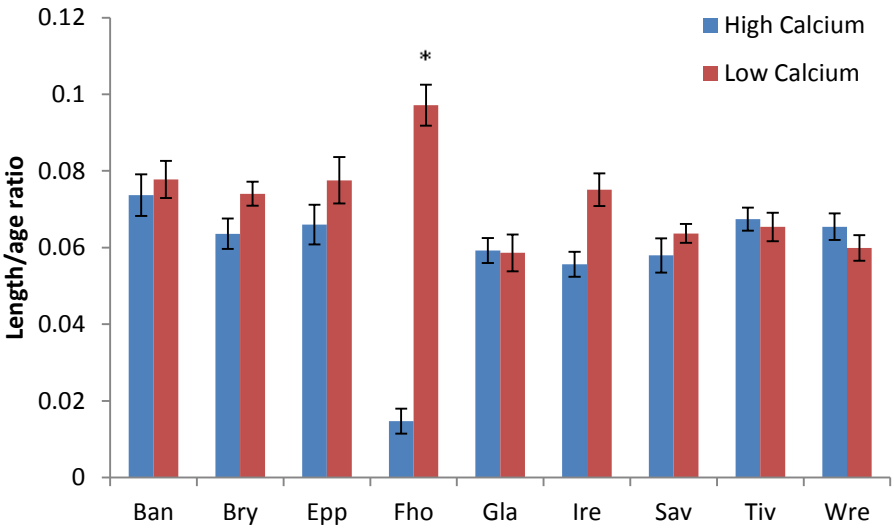


Figure 2.33. Mean hatchling group length/age ratio values for different populations and calcium treatments. Significant differences between high and low calcium treatment at the population level are indicated by asterisks (post- hoc Tukey test, P <0.05). Error bars display standard error of mean.

Isolated individuals

Table 2.11 displays the mean parameter terms of the Gompertz growth model (*a*, *b*, and *c*) for each of the F_2 populations with 95% confidence limits. The derived growth curves for each population (at high and low calcium treatment) are displayed in appendices 6.10 to 6.18.

Table 2.11. F_2 Gompertz parameter values with confidence limits for F_2 isolated individuals.

Population	*Calcium*	Mean parameter term		
		***a* (± 95% C.I.)**	***b* (± 95% C.I.)**	***c* (± 95% C.I.)**
Bank	Hi	30.27 (27.67, 33.20)	5.29 (3.96, 6.26)	0.985 (0.984, 0.986)
	Lo	25.01 (22.96, 27.11)	5.23 (3.96, 6.26)	0.989 (0.984, 0.986)
Brychfa	Hi	25.83 (23.94, 27.55)	7.14 (5.60, 8.69)	0.986 (0.984, 0.987)
	Lo	27.19 (25.38, 29.22)	5.59 (4.30, 6.70)	0.986 (0.984, 0.988)
Epping	Hi	37.26 (32.85, 41.13)	4.18 (3.64, 4.73)	0.991 (0989, 0.992)
	Lo	28.60 (26.75, 29.98)	5.27 (3.94, 6.86)	0.987 (0.986, 0.989)
Fauldhouse	Hi	28.47 (27.05, 29.88)	7.66 (5.90, 9.56)	0.985 (0.983, 0.987)
	Lo	26.12 (25.93, 28.78)	4.37 (3.94, 5.66)	0.983 (0.981, 0.984)
Glanahafren	Hi	28.15 (25.44, 30.28)	7.44 (5.17, 9.56)	0.987 (0.984, 0.989)
	Lo	23.44 (21.46, 24.59)	8.99 (5.76, 12.10)	0.985 (0.983, 0.986)
Ireland	Hi	25.87 (23.24, 29.00)	7.90 (5.10, 11.08)	0.987 (0.985, 0.988)
	Lo	23.08 (21.86, 24.61)	6.86 (4.76, 9.42)	0.984 (0.982, 0.986)
Savernake	Hi	26.27 (24.09, 27.93)	6.53 (5.36, 7.77)	0.986 (0.983, 0.987)
	Lo	24.86 (23.44, 26.06)	9.52 (7.04, 11.85)	0.984 (0.982, 0.985)
Tiv	Hi	28.04 (26.16, 28.28)	5.74 (4.60, 6.86)	0.987 (0.984, 0.988)
	Lo	27.85 (26.49, 29.54)	6.18 (4.60, 6.86)	0.986 (0.984, 0.988)
Wreake	Hi	28.57 (27.58, 29.72)	5.50 (4.47, 6.38)	0.987 (0.986, 0.988)
	Lo	24.25 (22.40, 25.85)	7.54 (6.17, 9.36)	0.985 (0.983, 0.986)

Analysis of F_2 growth curves:

Parameter a

The inverse reciprocal of parameter term *a* was taken to normalise the data and ANOVA performed. The results of the ANOVA are summarised in Table 2.12 and the raw mean parameter *a* values are displayed in Figure 2.34.

Table 2.12. Summary ANOVA table for F_2 parameter term *a* (inverse reciprocal).

Source	*D.F*	*F Statistic*	*P-value*	Significant
Population	8, 325	9.92	<0.001	Y
Calcium	1, 325	36.17	<0.001	Y
Population * Calcium	8, 325	3.87	<0.001	Y

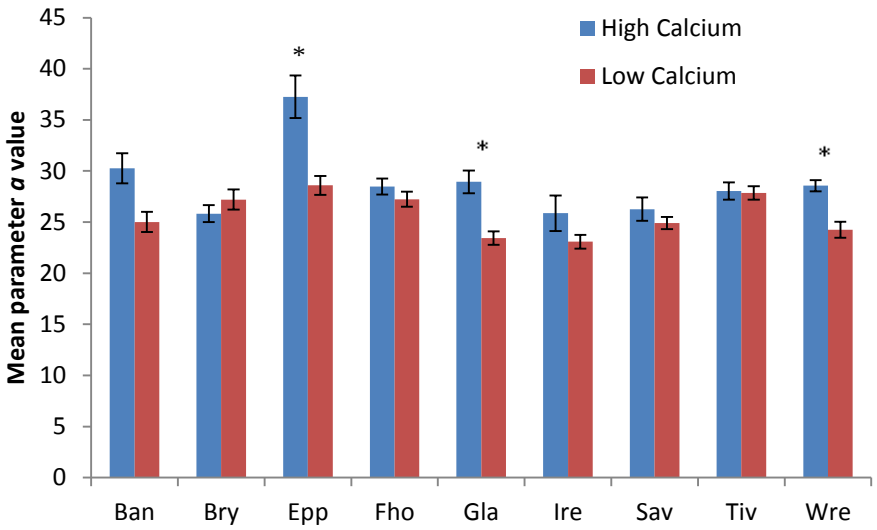


Figure 2.34. Mean parameter *a* values for different populations and calcium treatments. Significant differences between high and low calcium treatment are indicated by asterisks (post- hoc Tukey test, P <0.05). Error bars display standard error of mean.

Post-hoc Tukey tests revealed that the Epping, Glanahafren and Wreake populations displayed a significant difference across calcium treatments ($p<0.05$).

Parameter b

Parameter *b* was subjected to natural log transformation before being subjected to ANOVA analysis. The results of the analysis are displayed in Table 2.13 while Figure 2.35 displays the raw, mean values of parameter *b*.

Table 2.13. Summary ANOVA table for F_2 parameter term b (natural log transformed).

Source	*D.F*	*F Statistic*	*P-value*	Significant
Population	8, 325	2.78	0.005	Y
Calcium	1, 325	0.14	0.771	N
Population * Calcium	8, 325	2.89	0.004	Y

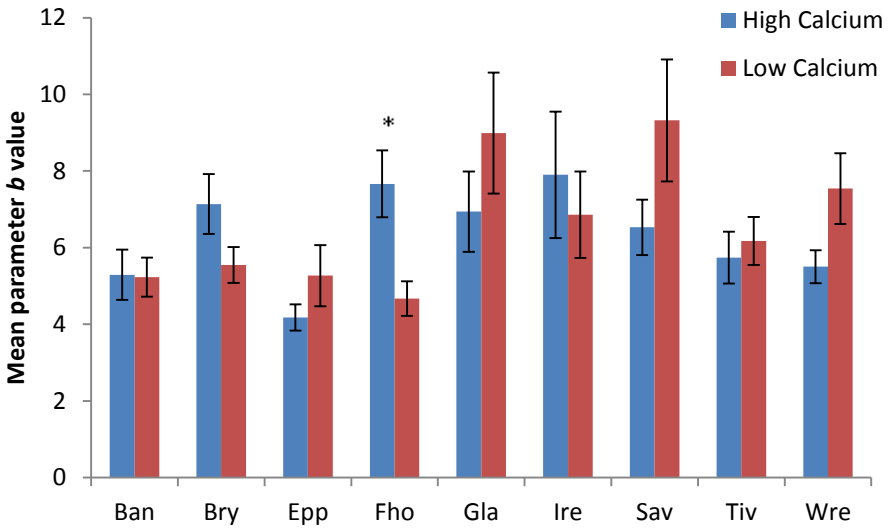


Figure 2.35. Mean parameter *b* values for different populations and calcium treatments. Significant differences between high and low calcium treatment are indicated by asterisks (post- hoc Tukey test, P <0.05). Error bars display standard error of mean.

Intra-population differences between calcium treatment were only found in the Fauldhouse population (post hoc Tukey test, p <0.05).

Parameter c

Parameter *c* values were found to be normally distributed before being subjected to ANOVA analysis. The results of the analysis are displayed in Table 2.14 while Figure 2.36 displays the mean values of parameter *c*.

Table 2.14. Summary ANOVA table for F_2 parameter term *c*.

Source	D.F	*F Statistic*	*P-value*	Significant
Population	8, 325	5.89	<0.001	Y
Calcium	1, 325	33.22	<0.001	Y
Population * Calcium	8, 325	1.35	0.216	N

Significant differences in parameter c values were found at the populations and calcium treatment levels, however no interaction was found between these terms.

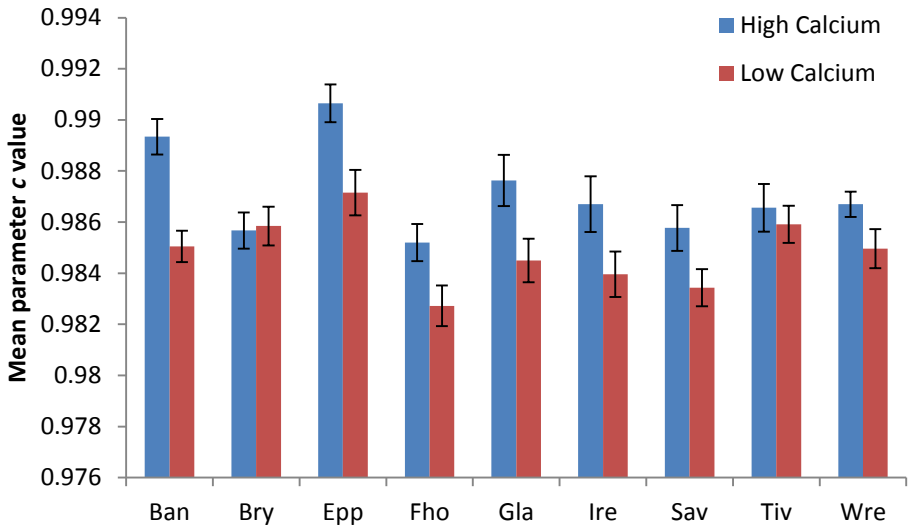
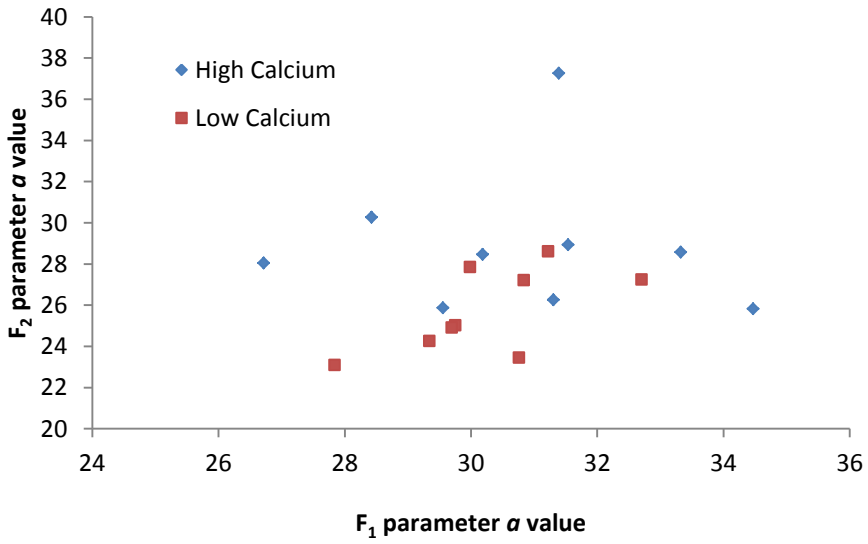


Figure 2.36. Mean parameter *c* values for different populations and calcium treatments. Error bars display standard error of mean.

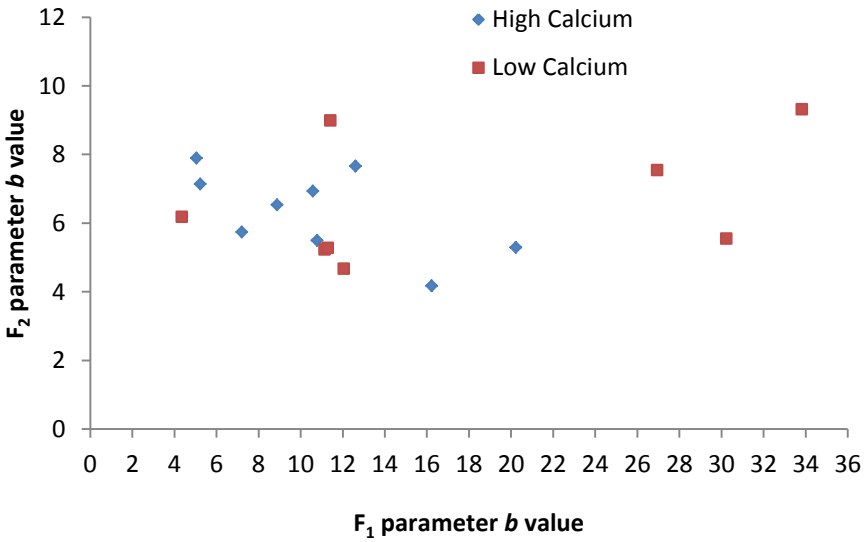
2.4.2.3. Inter-generational comparisons: Growth

Mean Gompertz parameter values for each population and calcium treatment from the F_1 and F_2 generations were plotted against each other. Figure 2.37 (i-iii) displays mean parameter values for each parameter term (*a*–*c*) and calcium treatment for the F_1 and F_2 generations. Pearson's product –moment correlation was performed to compare overall F_1 and F_2 mean values for each parameter. No parameter terms displayed a statistically significant correlation (P>0.05).

i.



ii.



iii.

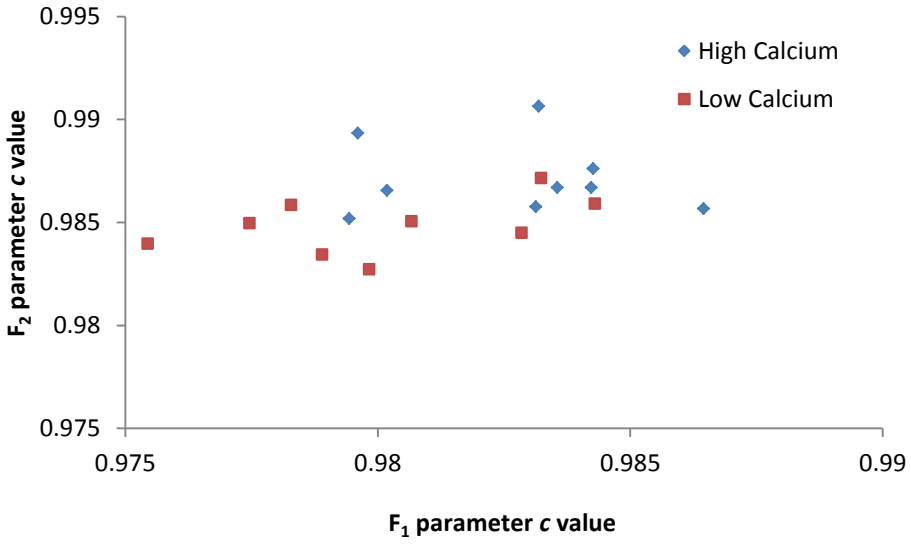


Figure 2.37. Scatter plots (i-ii) displaying F_1 and F_2 parameter comparisons for terms *a-c* by high and low calcium treatment.

When analysed separately the mean parameter *a* values for the low calcium treatment groups displayed a marginally non-significant correlation (n=9, r=0.641, P=0.063).

Differences in mean parameter values between generations were assessed by performing a paired t-test for each parameter the results of which are displayed in Table 2.15.

Table 2.15. Results of paired t-tests for main parameter terms across generations.

Parameter	Generation	Mean	St Dev	t-value	P-value	Significant
a	F_1	30.50	1.90	3.97	0.001	Y
	F_2	27.28	3.21			
b	F_1	16.07	12.23	3.37	0.004	Y
	F_2	6.47	1.44			
c	F_1	0.981	0.00292	-6.195	<0.001	Y
	F_2	0.986	0.00195			

From Table 2.15 it can be seen that all parameter terms were found to be significantly different across the F_1 and F_2 generations. Parameter *a* was found to be significantly lower in the F_2 generation indicating that the snails were generally larger in the F_1 generation than in the F_2 generation. Parameter *c* was shown to be significantly larger in the F_2 generation indicating that the snails were growing faster in the second generation. Initial size (parameter *b*) was shown to be larger in the F_1 generation.

2.4.3. Survivorship

2.4.3.1. F_1 survivorship

F_1 hatchling groups survivorship

Population was found to significantly affect F_1 hatchling group survivorship ($H = 15.90$, $DF = 8$, $P = 0.044$ (adjusted for ties)), while calcium treatment had no overall effect on mortality ($P = 0.945$).

Boxplots of the median percentage of surviving individuals for each population are displayed in Figure 2.38.

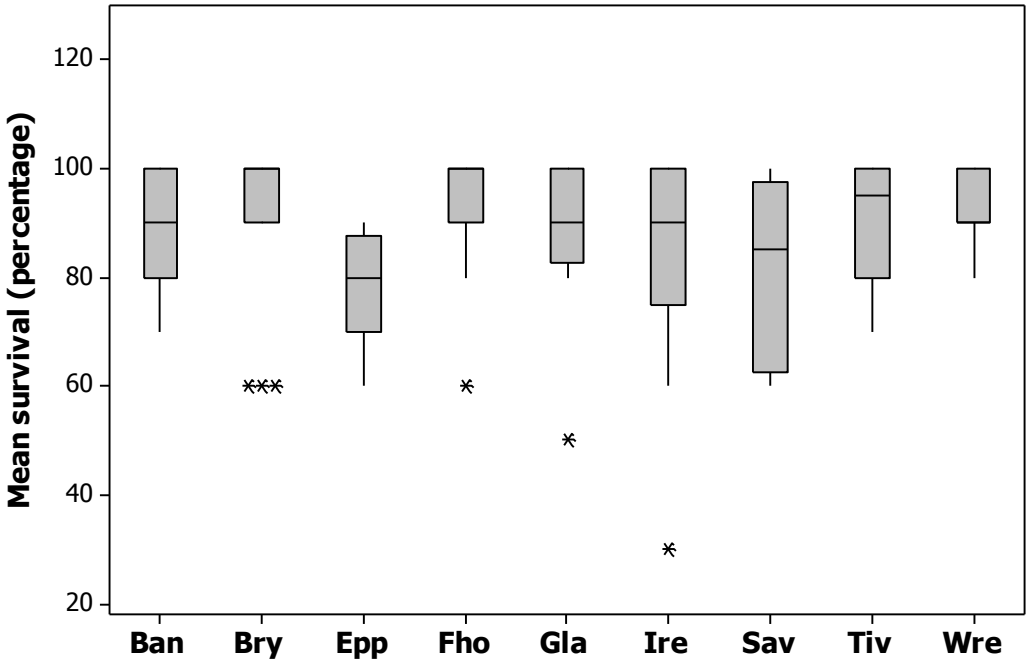


Figure 2.38. Boxplot of percentage survival for each population. Boxplots display median and inter-quartile range (IQR).

Individual Survivorship

Survivorship curves for all populations in high and low calcium treatments are displayed in Figure 2.39. All culled individuals (both reproductive and non-reproductive) were censored from analysis as were two incidents of mass mortality that occurred as a result of drowning due to the cups not being righted. The comparison of overall survivorship revealed a significant difference between the treatment groups (Wilcoxon log-rank, X^2=111.78, DF=17, P<0.001). Calcium alone was not found to have a significant effect on mortality (Wilcoxon log-rank, X^2=0.0178, DF=1, P=0.894)

In order to determine whether or not any inter-population differences in mortality existed, F_1 and F_2 generations for each population were subjected to pairwise survivorship analysis (Table 2.16).

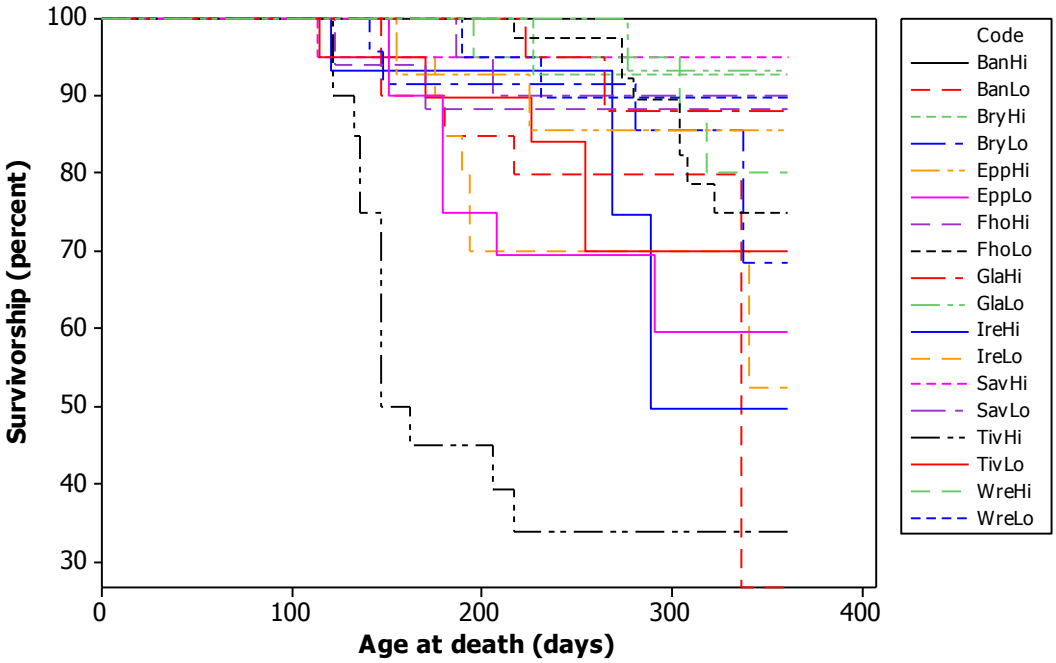


Figure 2.39. Survivorship curve for all populations and high (Hi) and low (Lo) calcium treatment combinations F_1 generation. All populations and treatment separate.

Due to the use of multiple comparisons, a Bonferroni correction was applied, whereby the new significance level is calculated by dividing the old significance level by 9 (the number of comparisons applied) which requires that P-values must be lower than 0.0055 in order to be classified as significant (Zar, 1999). After the Bonferroni correction was applied, only the Tiverton population displayed a significant difference across calcium treatment, although the p-value obtained for Bank was also reasonably low (0.021) and the Bonferroni test is known to be a conservative test (Zar, 1999).

Table 2.16. Pairwise survivorship F_1 generation: Calcium treatment, DF=1 for each population .

Population	X^2	*P-value*	Significant
Bank	5.34	0.021	N
Brychfa	0.341	0.559	N
Epping	1.79	0.180	N
Fauldhouse	0.010	0.919	N
Glanahafren	0.390	0.532	N
Ireland	0.203	0.652	N
Savernake	0.282	0.596	N
Tiverton	**10.7**	**0.001**	**Y**
Wreake	0.00740	0.931	N

2.4.3.2. F_2 survivorship

F_2 hatchling group survivorship

The F_2 hatchling group percentage mortality data were arcsine transformed and ANOVA performed. A summary of the ANOVA results is displayed in Table 2.17 and the raw mean mortality values are displayed in Figure 2.40.

Table 2.17. Summary ANOVA table for F_2 x10 mortality (arcsine square root transformed).

Source	D.F	F Statistic	P-value	Significant
Population	8, 102	5.64	<0.001	Y
Calcium	1, 102	0.41	0.525	N
Population * Calcium	8, 102	0.45	0.890	N

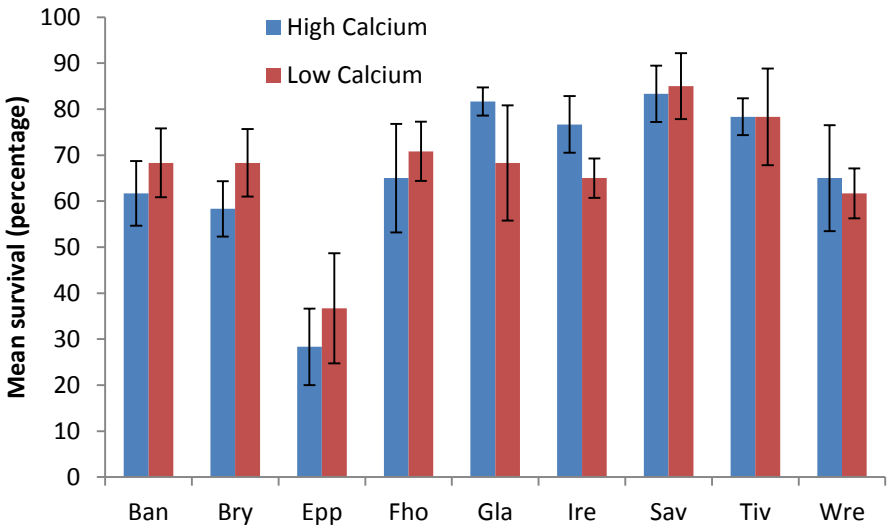


Figure 2.40. Mean percentage survivorship for F_2 x10 replicates.

No significant differences were found in survivorship between calcium treatment at the population level. The highest mortality occurred in the Epping population which was found to be significantly different from all other populations (post hoc Tukey Test, $p<0.05$).

Individual Survivorship

The survivorship curve for all F_2 populations at high and low calcium is displayed in Figure 2.41. All culled individuals (both reproductive and non-reproductive) were censored from analysis as before. The comparison of overall survivorship revealed a significant difference between the treatment groups (Wilcoxon log-rank, DF=17, X^2=69.09, P<0.001). Calcium treatment

alone was found to have a significant effect on mortality (Wilcoxon log-rank, DF=1, X^2=14.21, P<0.001, see Figure 2.42) In order to determine whether any inter-population differences in mortality existed, each population was subjected to pairwise survivorship analysis (with Bonferroni correction setting the new significance value to P=0.0055) and the results are displayed in Table 2.18.

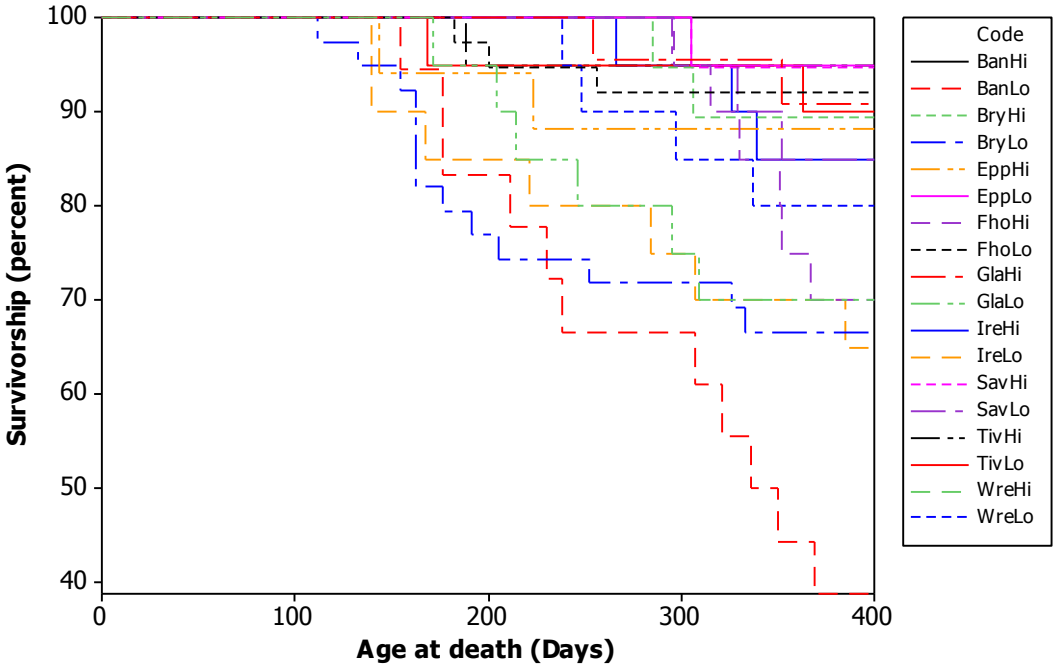


Figure 2.41. Survivorship curve F_2 generation. All populations and treatments separate.

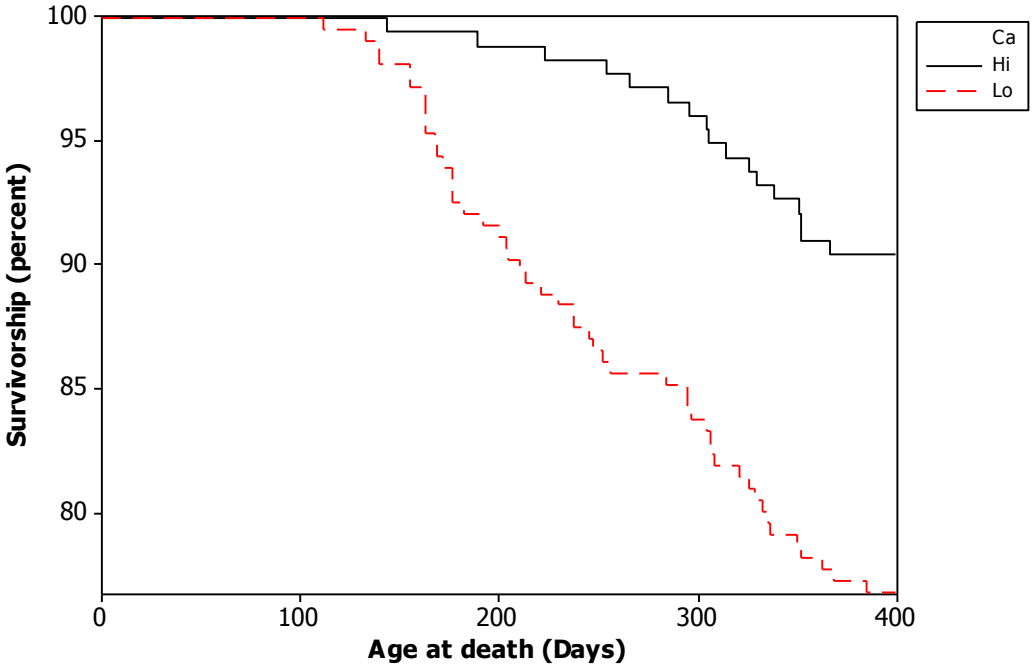


Figure 2.42. Survivorship curve F_2 generation comparing High and Low calcium treatments, all populations combined.

Table 2.18. Pairwise survivorship F_2 generation: Calcium treatment.

Population	X^2	*DF*	*P-value*	Significant
Bank	**16.92**	**1**	**<0.001**	**Y**
Brychfa	3.87	1	0.049	N
Epping	0.651	1	0.420	N
Fauldhouse	3.82	1	0.051	N
Glanahafren	3.42	1	0.640	N
Ireland	2.63	1	0.105	N
Savernake	0.921	1	0.337	N
Tiverton	0.351	1	0.554	N
Wreake	4.32	1	0.038	N

2.4.3.3. Inter-generational comparisons: Survivorship

No significant correlation was found between hatchling group survivorship for populations when compared across generations via Pearson's product moment correlation after arcsine conversion for percentages (n=18, r=0.228, P=0.556). The converted percentage data were then subjected to a 2-sample t-test and hatchling group mortality was found to be significantly higher in the

F_2 generation (DF=11, T=4.43, P=0.001) with approximately 20% lower survival being displayed in the F_2 hatchling groups.

2.4.4. Reproduction:

2.4.4.1. F_1 reproduction

Age at first reproduction

Age at first reproduction was found to not be normally distributed and was transformed by square root to yield normal data. ANCOVA (with age at isolation as a covariate) analysis of the data showed that there was a significant difference in age at first reproduction at the population and treatment levels, with a significant interaction between factors (Table 2.19). The overall trend suggested that populations took longer to reach first reproduction in the low calcium treatment although as the significant interaction term suggests, this effect was not consistent for all populations.

Table 2.19. Summary ANCOVA table for square root F_1 age at 1st reproduction.

Source	*D.F*	*F Statistic*	*P-value*	Significant
Sqrt age isolation	1, 249	2546.11	<0.001	Y
Population	8, 249	112.71	<0.001	Y
Calcium	1, 249	48.17	<0.001	Y
Population * Calcium	8, 249	30.99	<0.001	Y

The Brychfa, Ireland and Savernake populations showed a significant difference between calcium regime (Tukey test, P<0.05) with all three populations taking longer to achieve first reproduction in the low calcium treatment.

The raw mean ages of first reproduction for all populations and calcium treatments are displayed in Figure 2.43.

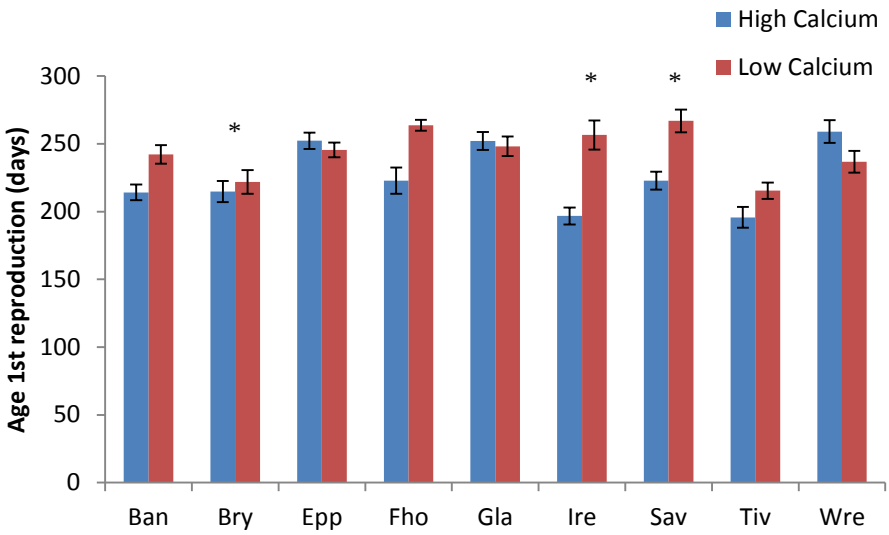


Figure 2.43. Age 1st reproduction: F_1 generation for all populations and calcium treatment. Populations marked with an asterisk display a significant difference across calcium treatment (post-hoc pairwise comparisons, P>0.05). Error bars display standard error of mean.

In order to gain more insight into any effects of calcium regime each treatment group was analysed independently, again with age at isolation as a covariate. The ranked ages at first reproduction for the low and high calcium groups are displayed in Figures 2.44 and 2.45 respectively.

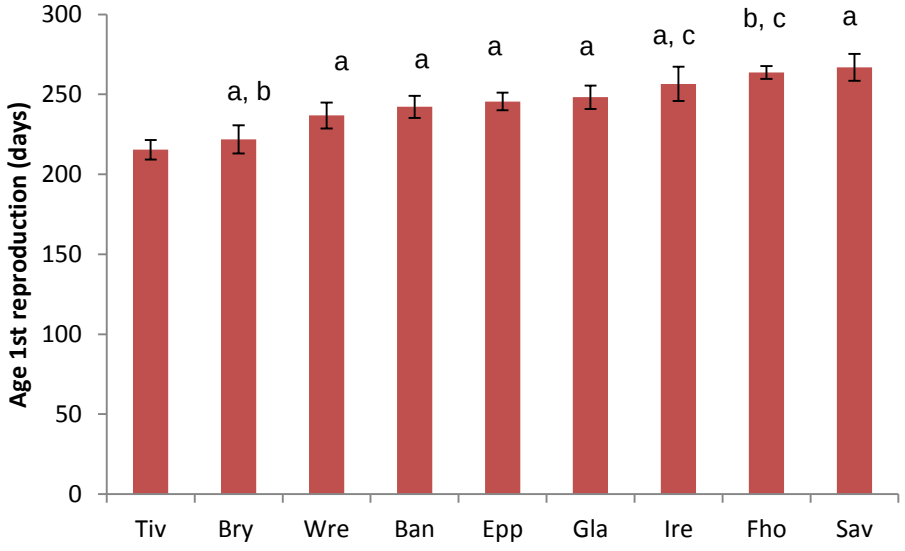


Figure 2.44. Rank age 1st reproduction: F_1 generation, low calcium treatment. Populations with the same letter have no significant difference between them (Post-hoc pairwise comparisons, P>0.05).

Age at isolation was found to be significant for the low calcium group (F=1304.83, DF, 1, 126, P<0.001), and significant differences between populations were also found (F=62.43, DF=8, 126, P<0.001 – see Figure 2.62). The ranked age of first reproduction for the high calcium group is shown below in Figure 2.45.

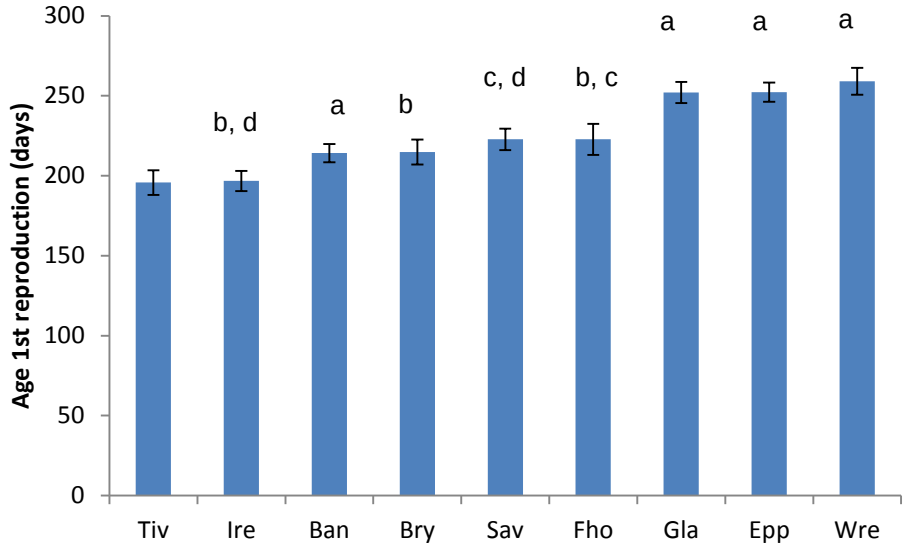


Figure 2.45. Rank age 1st reproduction: F_1 generation, high calcium treatment. Populations with the same letter have no significant difference between them (Post-hoc pairwise comparisons, P>0.05).

The covariate, age at isolation was also found to be significant for the high calcium group (F=1259.87, DF, 1, 122, P<0.001) and significant differences between populations were again found (F=89.69, DF=8, 122, P<0.001).

The extent to which changes in rank varied across calcium treatment varied with population. For example, some populations maintain the same ranked position (such as Tiverton), while others (e.g. Wreake, Savernake and Ireland) change rank dramatically between calcium treatment.

Size at first reproduction

ANCOVA (with age at isolation as a covariate) analysis of the size at first reproduction showed that there was a significant difference in size at first

reproduction at the population and treatment levels, with a significant interaction between factors (Table 2.20).

Table 2.20. Summary ANCOVA table for F_1 shell length at 1st reproduction.

Source	D.F	F Statistic	P-value	Significant
Age isolation	1, 244	35.35	<0.001	Y
Population	8, 244	13.59	<0.001	Y
Calcium	1, 244	31.49	<0.001	Y
Population * Calcium	8, 244	3.43	0.001	Y

Post-hoc Tukey tests revealed that overall the low calcium populations were significantly larger than the high calcium populations at the time of first reproduction. Both the Fauldhouse and Brychfa populations were found to display significant intra-population differences, with snails in the high calcium treatment groups found to be significantly smaller than those in the low calcium treatment groups at the onset of reproduction (post-hoc Tukey test, $p<0.05$, see Figure 2.46).

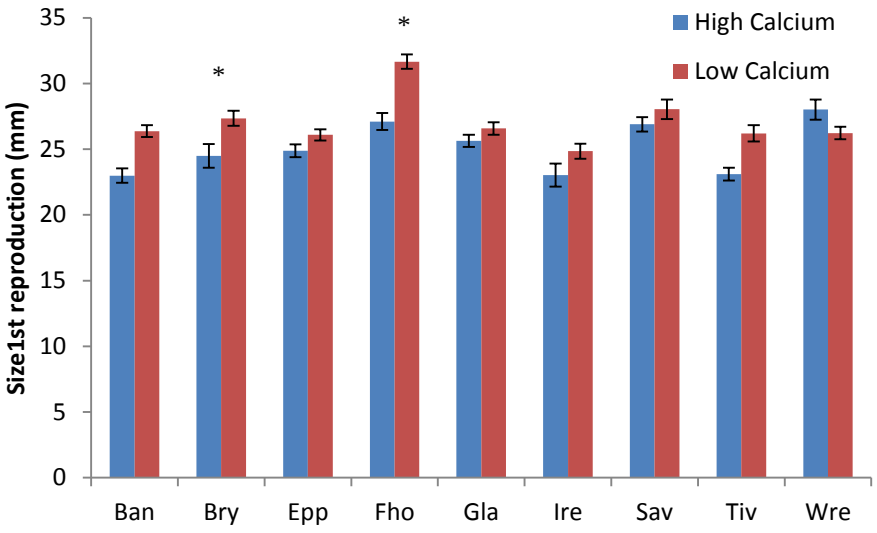


Figure 2.46. Size at 1st reproduction: F_1 generation for all populations and calcium treatment. Populations marked with an asterisk display a significant difference across calcium treatment (post-hoc pairwise comparisons, P<0.05). Error bars display standard error of mean.

Number of egg masses

The total number of egg masses produced in 21 days after the onset of reproduction was square root transformed and analysed using ANOVA. The

results of this analysis are summarised in Table 2.21, where neither population nor treatment were found to have a significant effect on the number of egg masses produced.

Table 2.21. Summary ANOVA table for F_1 total number of egg masses in 21 days.

Source	D.F	F Statistic	P-value	Significant
Population	8, 255	1.76	0.085	N
Calcium	1, 255	1.02	0.313	N
Population * Calcium	8, 255	1.28	0.255	N

Number of eggs

The total number of eggs produced in 21 days after the onset of reproduction were square root transformed and analysed using ANOVA. The results of this analysis are summarised in Table 2.22.

Table 2.22. Summary ANOVA table for F_1 square root total number of eggs in 21 days.

Source	***D.F***	***F Statistic***	***P-value***	**Significant**
Population	8, 255	2.43	0.015	Y
Calcium	1, 255	0.35	0.555	N
Population * Calcium	8, 255	1.60	0.126	N

Figure 2.47 displays the ranked raw mean values for the total number of eggs produced for each population, with calcium treatments combined. Significant inter-population differences in the number of eggs produced were found between the Epping population and the Ireland and Bank populations, where Epping was found to produce significantly more eggs than the latter two populations.

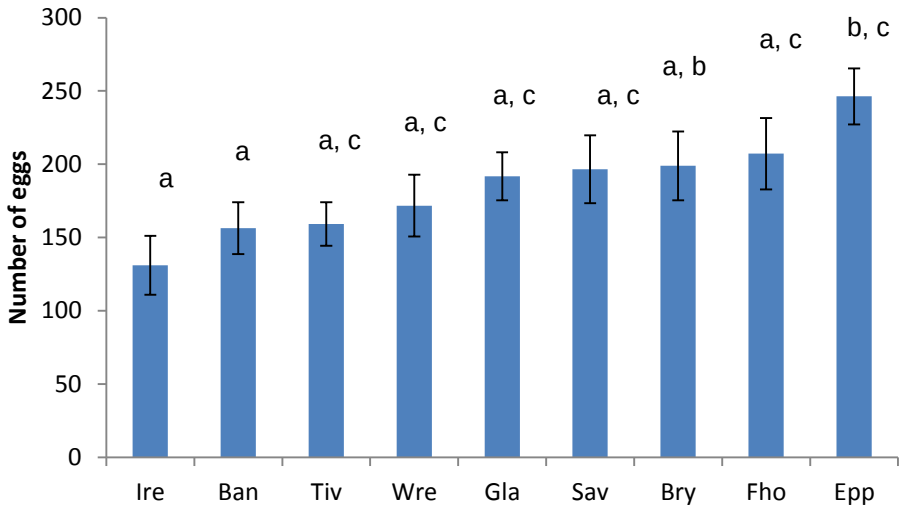


Figure 2.47. Ranked mean values (for both high and low calcium treatments combined as there was no significant effect of calcium on total egg number) for total eggs produced in 21 days after 1st onset of reproduction. Populations with the same letter have no significant difference between them (Post-hoc pairwise comparisons, P>0.05).

Eggs per mass

The mean number of eggs per mass produced in 21 days after the onset of reproduction were analysed using ANOVA. The data were found to be normal and no transformation was required. The results of this analysis are summarised in Table 2.23. A significant difference at the population level was found with the Epping population again being found to differ significantly from the Bank, Brychfa and Ireland populations by producing more eggs per mass. The significant interaction term suggests that some populations differ in their response to calcium but the lack of significance in the Calcium term indicates that this trend is not conserved throughout the populations. The mean eggs per mass values for each population and calcium treatment are displayed in Figure 2.48.

Table 2.23. Summary ANOVA table for F_1 mean eggs per egg mass.

Source	*D.F*	*F Statistic*	*P-value*	Significant
Population	8, 255	3.61	0.001	Y
Calcium	1, 255	0.13	0.719	N
Population * Calcium	8, 255	2.56	0.011	Y

Post-hoc pairwise comparisons of the high and low calcium data revealed that no significant intra-population differences in mean egg per mass could be

found despite the Fauldhouse and Savernake populations displaying a trend towards reduced eggs per mass in the low calcium treatment (Tukey Test, P>0.05 – See Figure 2.48).

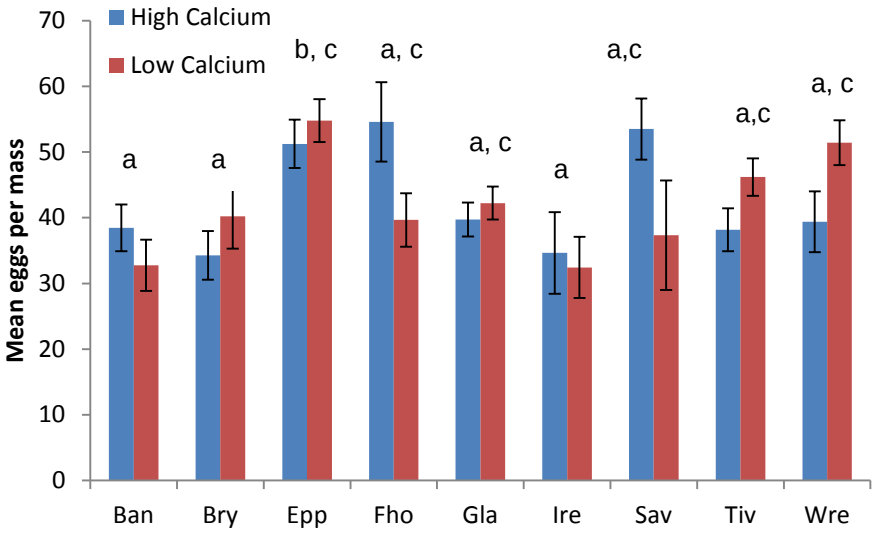


Figure 2.48. Mean eggs per mass for each population and calcium treatment. Populations with the same letter have no significant difference between them (Post hoc Tukey Test, P<0.05). Error bars denote standard error of mean.

Egg survivorship

Proportional egg survivorship values were subjected to arcsine transformation and analysed via ANOVA. The results of this analysis are displayed in Table 2.24. Significant differences in egg survivorship were found at the population level (although a post hoc Tukey test could not confirm where these differences lay) and a significant interaction between calcium treatment and population was found. Post hoc pairwise comparisons (Tukey Test P<0.05) did not reveal any significant intra-population differences across calcium treatment.

Figure 2.49 displays the ranked mean percentage egg survivorship for the populations studied.

Table 2.24. Summary ANOVA table for F_1 arcsine transformed mean survival to hatching.

Source	D.F	F Statistic	P-value	Significant
Population	8, 233	2.39	0.017	Y
Calcium	1, 233	0.09	0.765	N
Population * Calcium	8, 233	2.71	0.007	Y

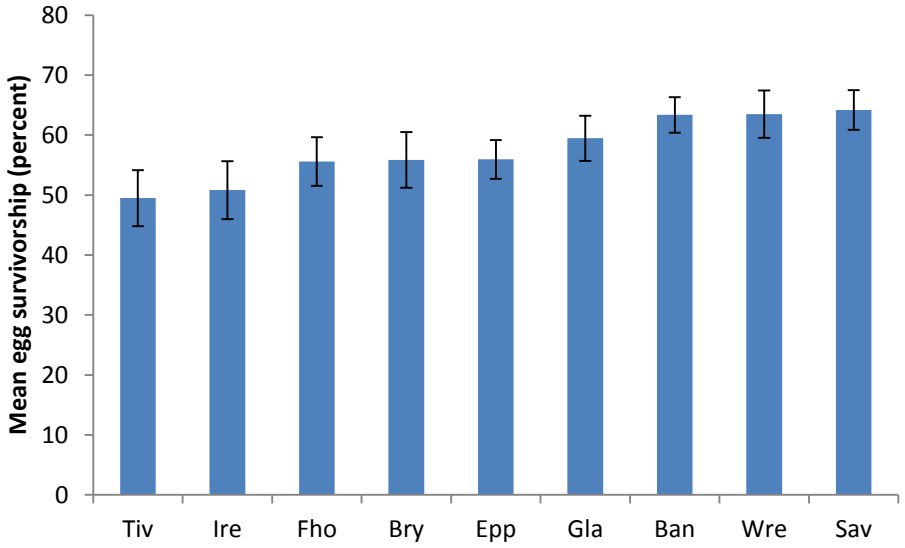


Figure 2.49. Rank mean egg survivorship values for study populations. Post-hoc Tukey Tests did not reveal a significant difference across the populations or at the intra population level (P<0.05). Error bars denote standard error of mean.

2.4.4.2. F_2 reproduction

Age at first reproduction

By the end of the study, some individuals (particularly those from the low calcium Fauldhouse population, where only 7.5% of individuals had reproduced by the time of the final cull) had failed to reproduce and were finally culled on the 28th of August 2009. As it was assumed that these individuals would eventually reproduce, initial analysis attempted to assign them an arbitrary age of first reproduction as the date they were culled. However, this resulted in data that were not appropriate for a parametric test and it was decided to remove all the non-reproducing individuals and the Fauldhouse population completely (which was most affected) for comparison of the high and low treatment groups.

Analysis of the high calcium data alone was carried out separately (with age at isolation as a covariate DF= 1, 129, F=14.78, P<0.001) and a significant difference was found at the population level (DF, 8. 129, F=4.09, p<0.001), with the Fauldhouse population being found to take significantly longer to reproduce than the Ireland and Epping populations (post-hoc Tukey test, P<0.05 – See Figure 2.50). Given that the low calcium Fauldhouse group was taking even longer to reproduce it is likely that the differences detected in the timing of first reproduction would have been greater if the low calcium treatment group was able to be included in the analysis. The ranked age at first reproduction data for the high calcium treatment group is displayed in Figure 2.68.

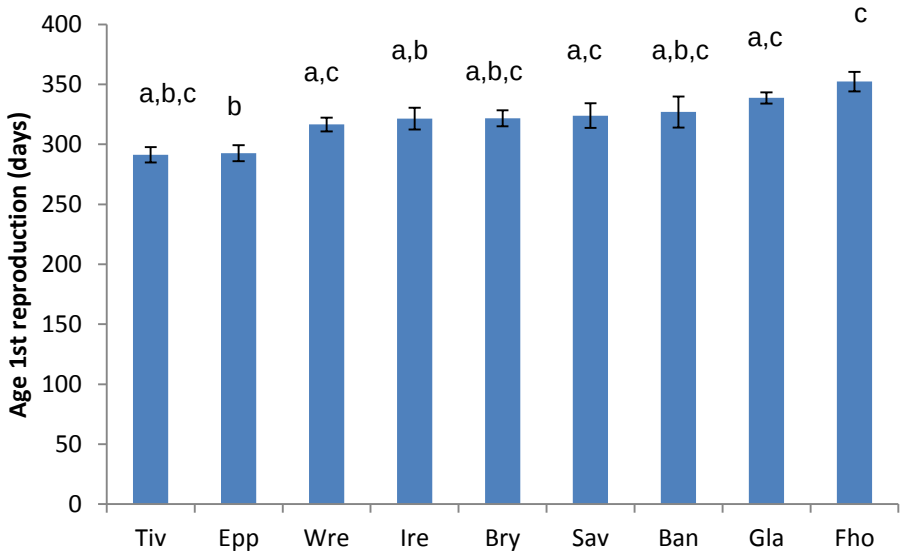


Figure 2.50. Rank age 1st reproduction: F_2 generation, high calcium treatment. Populations with the same letter have no significant difference between them (Tukey test, P<0.05). Error bars denote standard error of mean.

The low calcium treatment group (with the Fauldhouse population removed) was also analysed via ANCOVA and is shown in Figure 2.51. The covariate age at isolation was found to be significant (DF= 1, 103, F=32.04, P<0.001) and a significant difference was found at the population level (DF, 7. 103, F=4.80, p<0.001), with the Epping population being found to take significantly less time to reach first reproduction than the Ireland, Savernake and Wreake populations (post-hoc Tukey test, P<0.05 – see Figure 2.51).

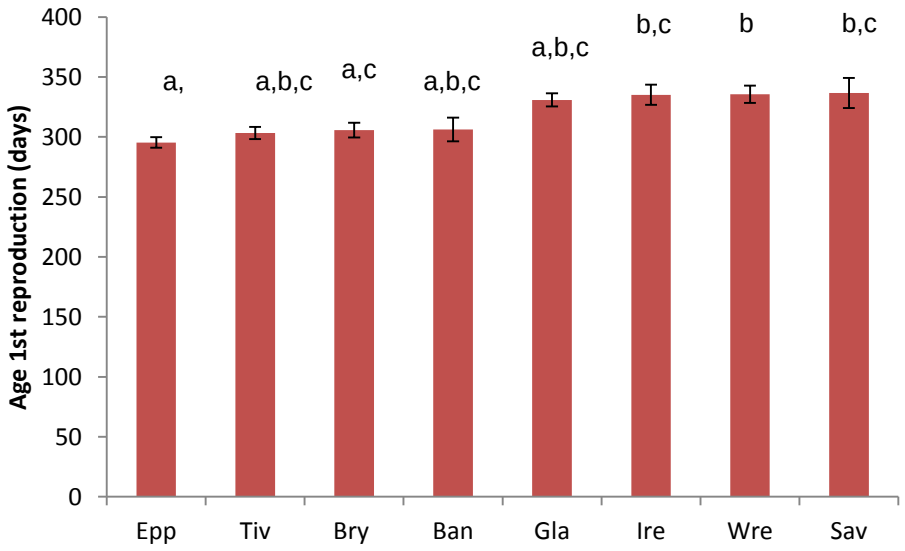


Figure 2.51. Rank age 1st reproduction: F_2 generation, low calcium treatment. Populations with the same letter have no significant difference between them (Post-hoc pairwise comparisons, P>0.05). Error bars denote standard error from mean.

Finally, ANCOVA was performed on the whole high and low calcium dataset from the remaining 8 populations with Fauldhouse removed, with age at isolation as a covariate and the data square root transformed to achieve normality. Analysis of the data showed that there was a significant difference in age at first reproduction at the population and treatment levels, with no interaction between factors (Table 2.25). As with the F_1 generation, the overall trend suggested that populations took longer to reach first reproduction in the low calcium treatment with the lack of a significant interaction term indicating that trend was consistent across all populations – although some reproduced later in high calcium the overall trend was for first reproduction to take longer in the low calcium treatments (see Figures 2.51 and 2.52).

Table 2.25. Summary ANCOVA table for square root F_2 age at 1st reproduction.

Source	*D.F*	*F Statistic*	*P-value*	Significant
Sqrt age isolation	1, 223	38.14	<0.001	Y
Population	7, 223	6.51	<0.001	Y
Calcium	1, 223	7.07	0.008	Y
Population * Calcium	7, 223	0.65	0.715	N

No intra-population differences in age at first reproduction were found in response to calcium treatment (post-hoc Tukey test, P>0.05). The raw mean ages of first reproduction for all populations and calcium treatments are displayed in Figure 2.52.

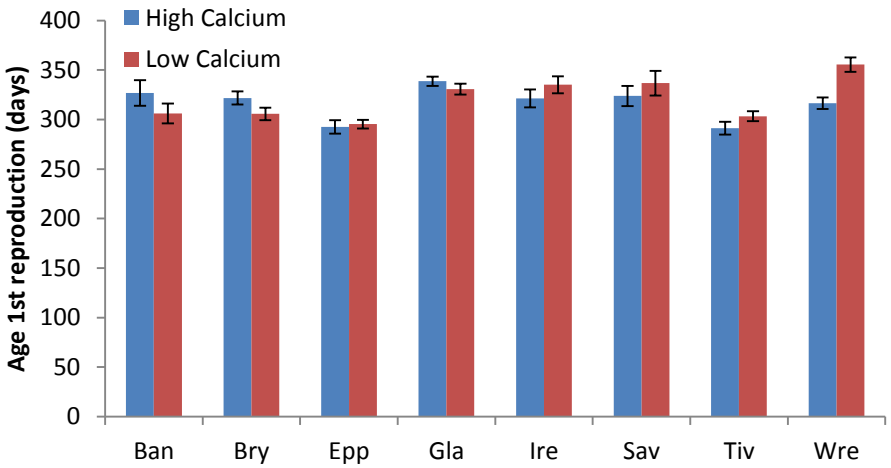


Figure 2.52. Age 1st reproduction: F_2 generation for all populations and calcium treatment (Fauldhouse excluded). No intra-population differences were found in response to calcium treatment (post-hoc pairwise comparisons, P>0.05). Error bars denote standard error from mean.

Size at first reproduction

Age at isolation was not found to be a significant covariate and subsequent ANOVA analysis of the size at first reproduction showed that there was a significant difference in size at first reproduction at the population level, with a significant interaction between factors. Calcium treatment alone was not found to have a significant effect on size at first reproduction (Table 2.26).

Table 2.26. Summary ANCOVA table for F_2 size at 1st reproduction.

Source	*D.F*	*F Statistic*	*P-value*	Significant
Population	8, 223	8.55	<0.001	Y
Calcium	1, 223	1.53	0.217	N
Population * Calcium	8, 223	3.64	0.001	Y

Post-hoc Tukey tests revealed that no significant intra-population differences could be found in size at first reproduction across different calcium treatments.

(post-hoc Tukey test, p>0.05). The ranked mean sizes at first reproduction for each population are displayed in Figure 2.53.

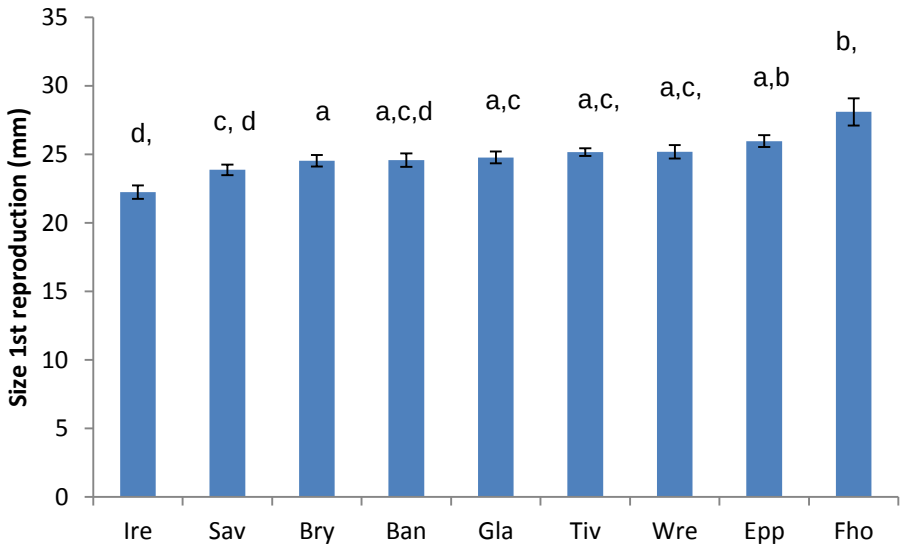


Figure 2.53. Ranked size 1st reproduction: F_s generation. Populations with the same letter have no significant difference between them (Post-hoc pairwise comparisons, P>0.05). Error bars denote standard error from mean.

The Fauldhouse population was found to be significantly larger at first reproduction than all but the Epping population, while the Ireland population was smaller at reproductive onset than all but the Savernake and Bank Well populations (Tukey test, p<0.05).

Number of egg masses

The total number of egg masses produced in 21 days after the onset of reproduction was analysed using ANOVA and the results of this analysis are summarised in Table 2.27. A significant difference in the total number of masses produced was found at the population level, while no difference was found at the treatment level. However the effects of calcium were approaching significance and there seemed to be a general trend towards greater mass production in the high calcium treatment. The ranked overall population mean data are displayed in Figure 2.54.

Table 2.27. Summary ANOVA table for F_2 total eggs masses in 21 days.

Source	D.F	F Statistic	P-value	Significant
Population	8, 248	4.46	<0.001	Y
Calcium	1, 248	2.98	0.085	N
Population * Calcium	8, 248	1.53	0.146	N

Post-hoc pairwise comparisons revealed that the Brychfa, Wreake and Glanahafren populations produced significantly more egg masses than the Tiverton and Savernake populations (see Figure 2.54).

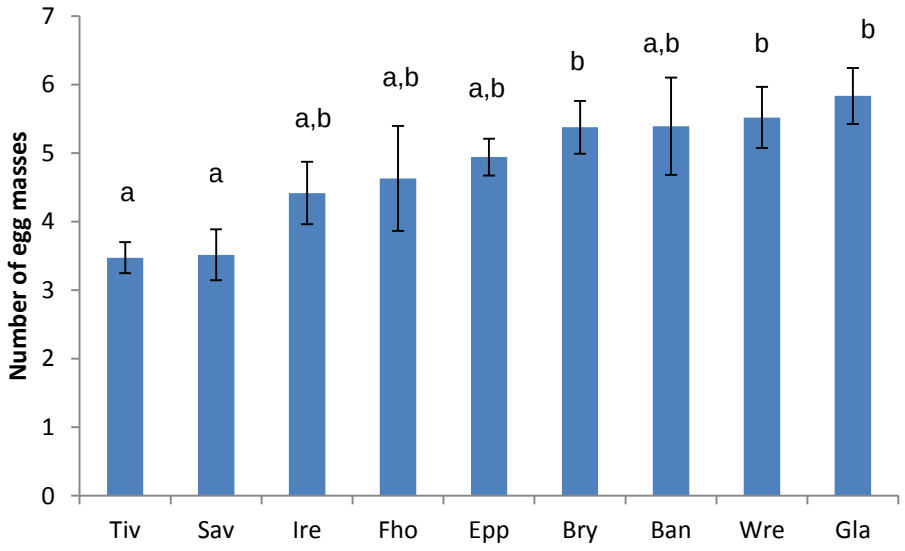


Figure 2.54. Ranked number of egg masses: F_2 generation for all populations. Populations with the same letter have no significant difference between them (Post-hoc pairwise comparisons, P>0.05). Error bars denote standard error of mean.

Number of eggs

The total number of eggs produced in 21 days was square root transformed prior to GLM ANOVA being performed and the results are displayed in Table 2.28. A significant difference in total eggs produced was found at the population level while no effect of calcium treatment was observed. The ranked population data are displayed in Figure 2.55.

Table 2.28. Summary ANOVA table for F_2 total eggs in 21 days (square root transformed).

Source	D.F	F Statistic	P-value	Significant
Population	8, 248	9.42	<0.001	Y
Calcium	1, 248	2.38	0.124	N
Population * Calcium	8, 248	1.00	0.434	N

Post-hoc pairwise comparisons (Tukey Test, P<0.05) revealed that Epping was found to produce significantly more eggs than all other populations and within the remaining eight populations the Savernake population produced significantly less eggs than the Tiverton and Wreake populations.

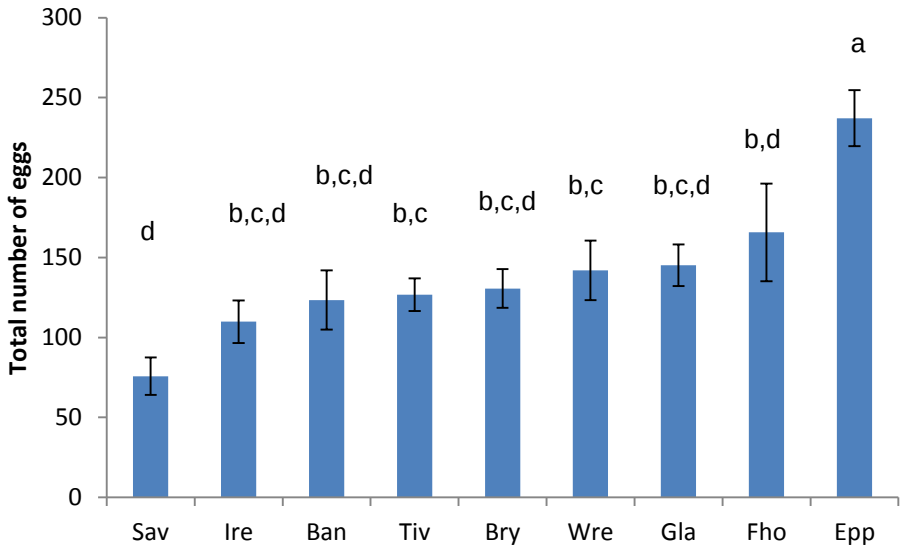


Figure 2.55. Ranked total number of eggs produced in 21 days: F_2 generation for all populations. Populations with the same letter have no significant difference between them (Post-hoc pairwise comparisons, P>0.05). Error bars denote standard error of mean.

Eggs per mass

The mean number of eggs per mass produced in 21 days after the onset of reproduction was analysed using ANOVA. The data were found to be normal and no transformation was required. The results of this analysis are summarised in Table 2.29. A significant difference at the treatment level was found, indicating that in general more eggs per mass were laid in the high calcium treatments.

Table 2.29. Summary ANOVA table for mean eggs per egg mass.

Source	*D.F*	*F Statistic*	*P-value*	Significant
Population	8, 237	13.22	<0.001	Y
Calcium	1, 237	10.00	0.002	Y
Population * Calcium	8, 237	0.93	0.491	N

A significant difference was also found at the population level with the Epping population being found to produce more eggs per mass than all but the Fauldhouse and Tiverton populations (See Figure 2.56). No significant difference in eggs per mass could be found at the intra-population level between calcium treatments (See Figure 2.57).

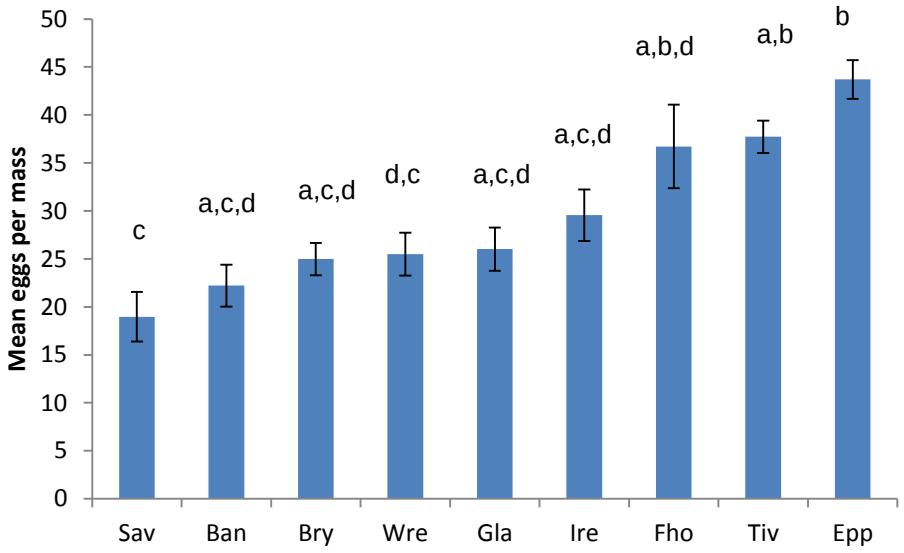


Figure 2.56. Ranked total number of eggs per mass produced in 21 days: F_2 generation for all populations. Populations with the same letter have no significant difference between them (Post-hoc pairwise comparisons, P>0.05). Error bars denote standard error of mean.

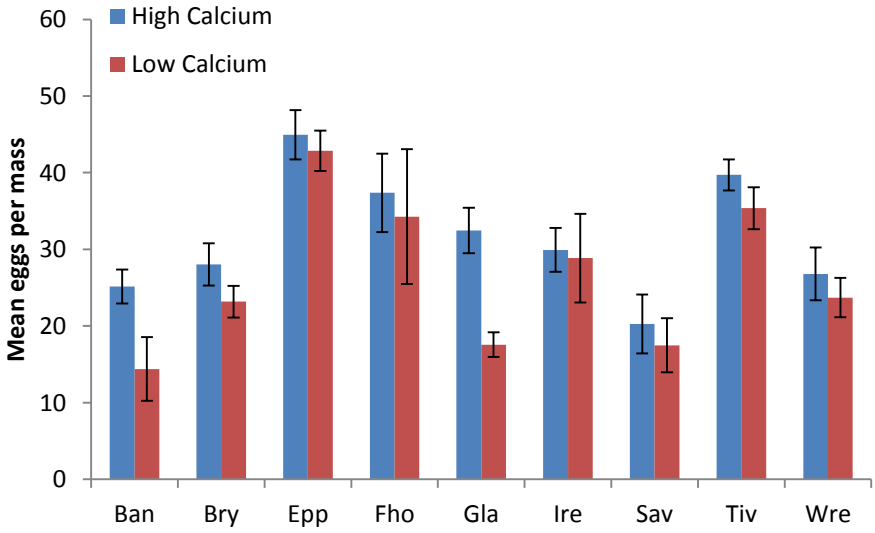


Figure 2.57. Mean eggs per mass in 21 days: F_2 generation for all populations and calcium treatment. Error bars denote standard error of mean.

Egg survival

Proportional egg survivorship values were subjected to arcsine transformation and analysed via ANOVA. The results of this analysis are displayed in Table 2.30. Significant differences in egg survivorship were found at the population level, while the effect of calcium on egg survivorship was not found to be significant. Egg survivorship was found to be significantly higher in the Bank population relative to the Brychfa, Savernake, and Tiverton populations (See Figure 2.58).

Table 2.30. Summary ANOVA table for egg survivorship to hatching.

Source	*D.F*	*F Statistic*	*P-value*	Significant
Population	8, 236	5.70	<0.001	Y
Calcium	1, 236	0.84	0.361	N
Population * Calcium	8, 236	1.42	0.189	N

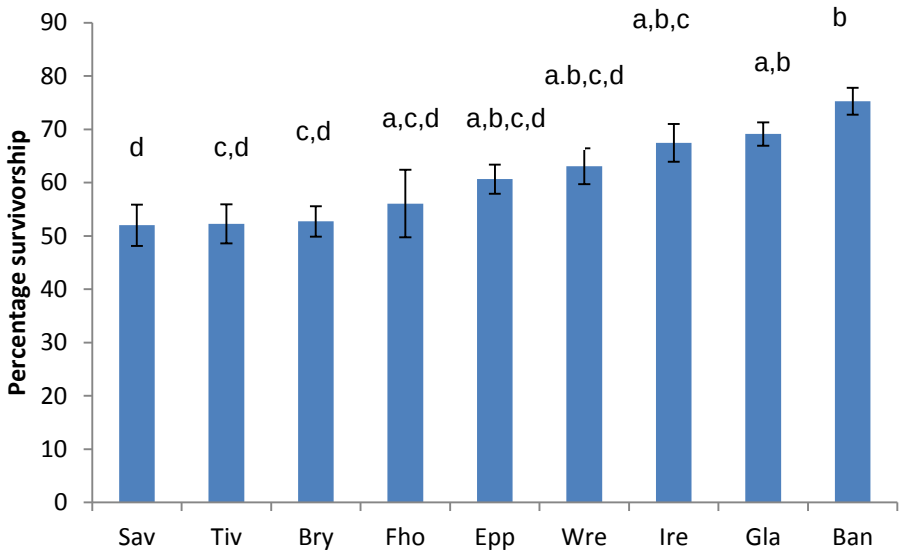


Figure 2.58. Ranked egg survivorship: F_2 generation for all populations. Populations with the same letter have no significant difference between them (Post-hoc pairwise comparisons, P>0.05). Error bars denote standard error from mean.

2.4.4.3. Inter-generational comparisons: Reproduction

Age at first reproduction

No significant correlation was found between the mean F_1 and F_2 ages at first reproduction when compared via Pearson's product moment correlation (n=18, r=0.065, P=0.797); see Figure 2.59.

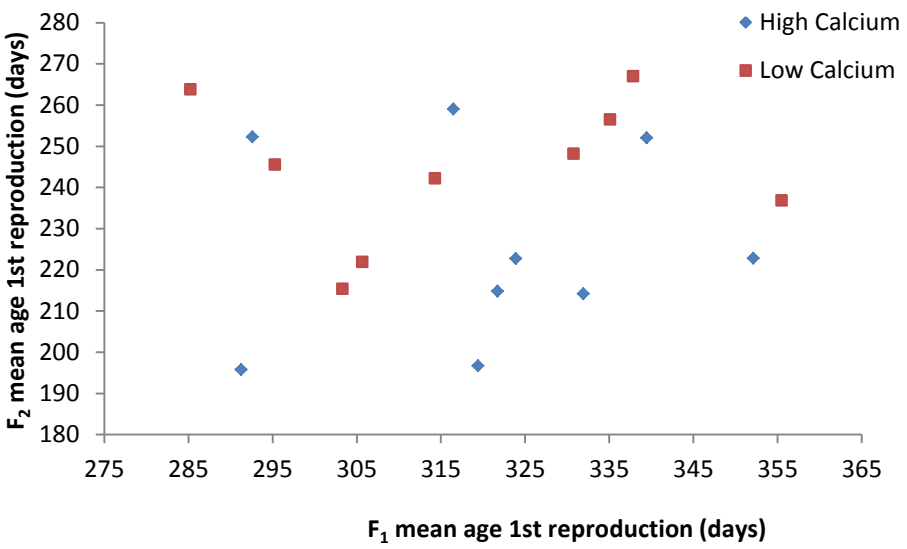


Figure 2.59. Scatter plot displaying age 1st reproduction across F_1 and F_2 generations by high and low calcium treatment.

Size at first reproduction

A significant correlation was found between the mean F_1 and F_2 sizes at first reproduction (Pearson's product moment correlation, n=18, r=0.581, P=0.011); see Figure 2.60. When analysed separately only the low calcium treatments displayed a significant correlation between F_1 and F_2 values (n=9, r=0.854, p=0.003).

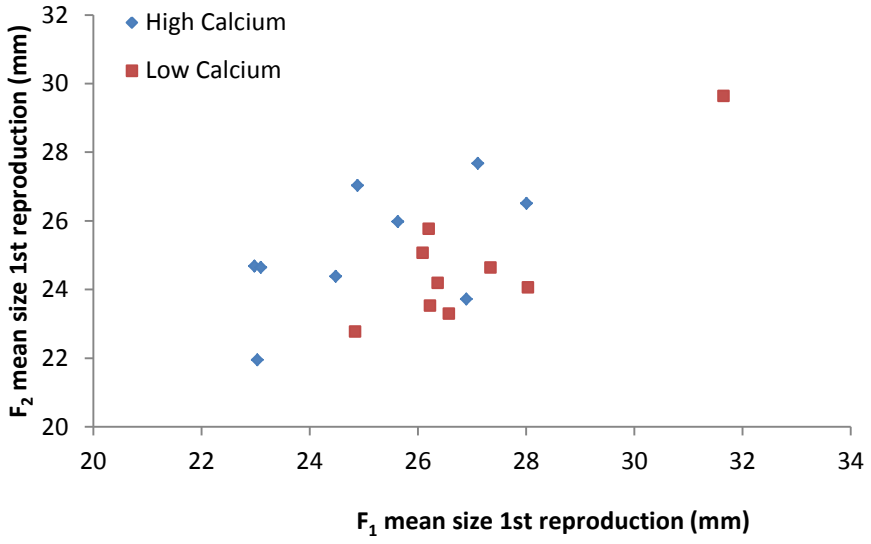


Figure 2.60. Scatter plot displaying size 1st reproduction across F_1 and F_2 generations by high and low calcium treatment

Egg Survivorship

Comparison of egg survivorship revealed no significant correlation between generations (Pearson's product-moment coefficient, n=18, r=0.312, P=0.207 – see Figure 2.61). No significant difference in egg survivorship was found between F_1 and F_2 generations (Paired t-test, P>0.05).

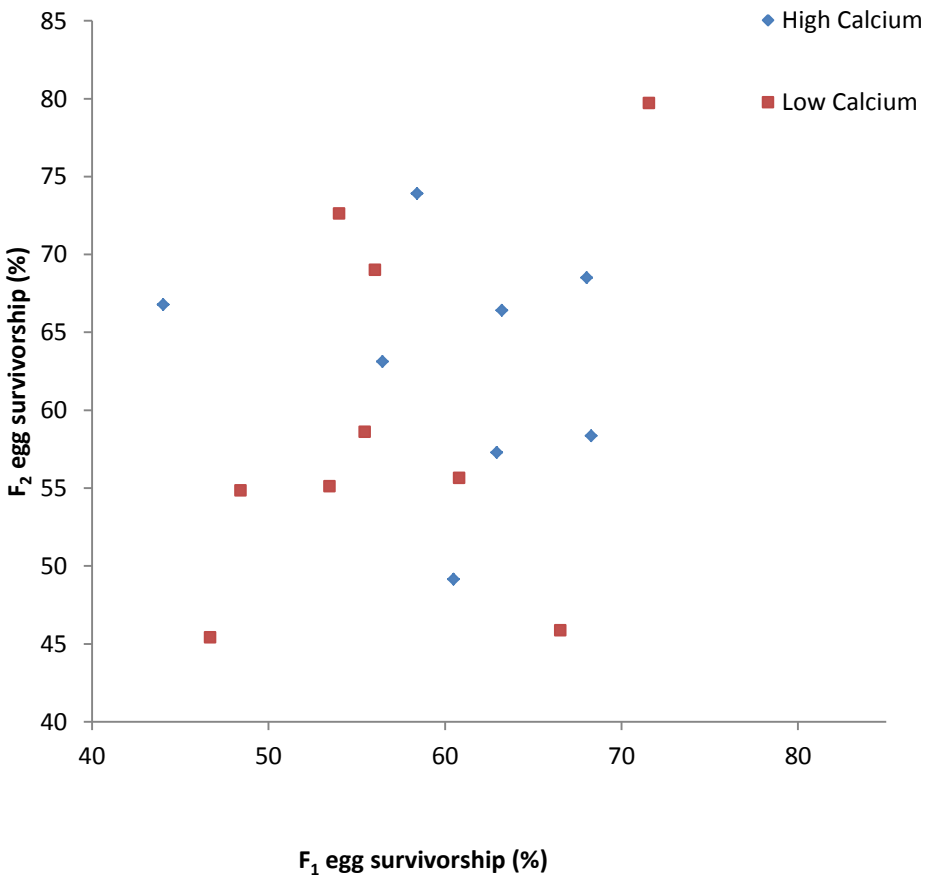


Figure 2.61. Scatter plot displaying mean percentage egg survivorship across F_1 and F_2 generations by high and low calcium treatment.

Reproductive output: Number of egg masses.

Comparison of the number of egg masses laid in the 21 day period revealed a significant correlation between generations (Pearson's product-moment coefficient, n=18, r=0.492, P=0.038 – see Figure 2.62). Across all populations, significantly more egg masses were laid in the F_2 generation than in the F_1

generation (Paired t-test, F_1 mean = 4.121± S.E. 0.169, F_2 mean = 4.861± S.E. 0.255, T = -3.27, P=0.004).

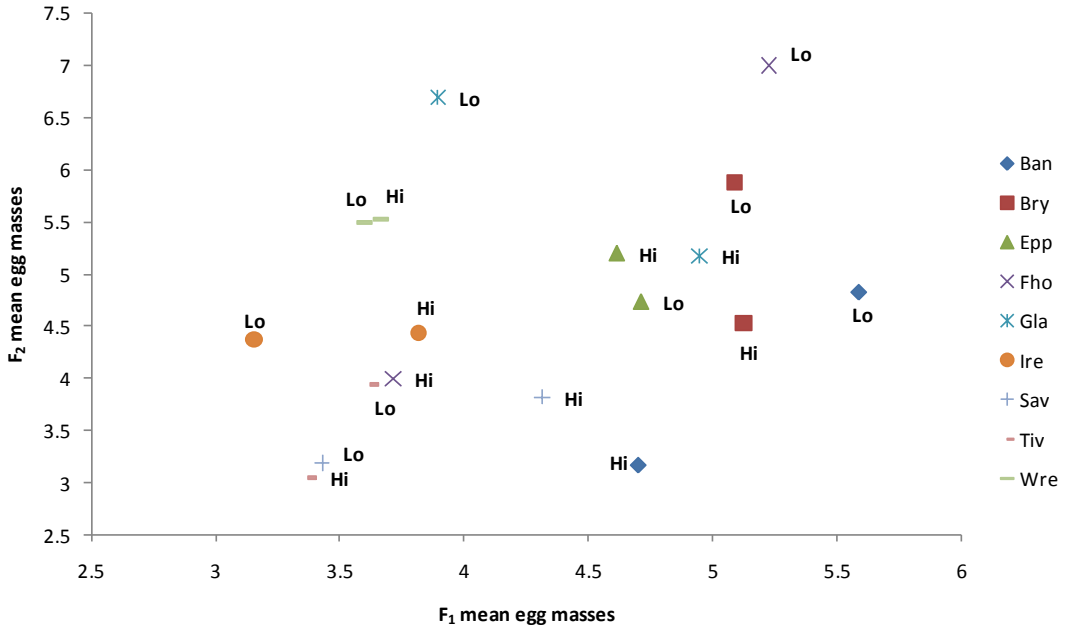


Figure 2.62. Scatter plot displaying mean number of egg masses across F_1 and F_2 generations by high and low calcium treatment and population.

Mean eggs per mass

Comparison of mean eggs per mass revealed no significant correlation between generations (Pearson's product-moment coefficient, n=18, r=0.448, P=0.063 – see Figure 2.63). Across all populations, significantly fewer eggs per mass were laid in the F_2 generation than in the F_1 generation (Paired t-test, F_1 mean = 42.27± S.E. 1.813, F_2 mean = 29.31± S.E. 2.339, T = 5.82, P<0.001).

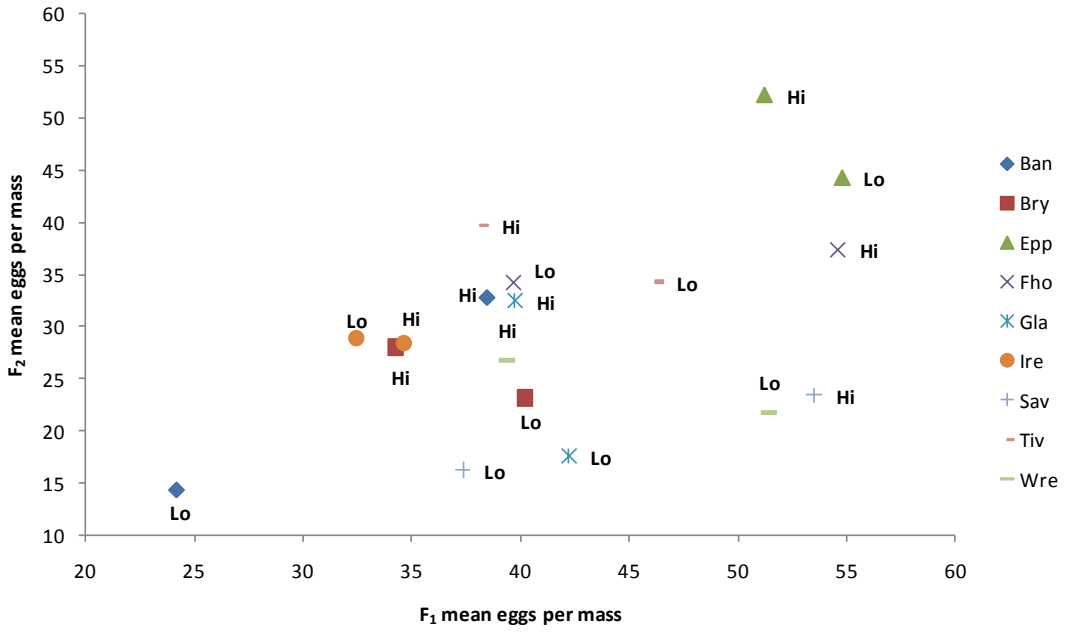


Figure 2.63. Scatter plot displaying mean eggs per mass across F_1 and F_2 generations by population and high and low calcium treatment.

Total eggs in 21 days

Comparison of total eggs produced over the 21 day period revealed a significant correlation between generations (Pearson's product-moment coefficient, n=18, r=0.625, P=0.006 – see Figure 2.64). Across all populations, significantly more eggs were laid in the F_1 generation than in the F_2 generation (Paired t-test, F_1 mean = 180.5± S.E. 9.827, F_2 mean = 139.9± S.E. 12.07, T = 4.19, P=0.001).

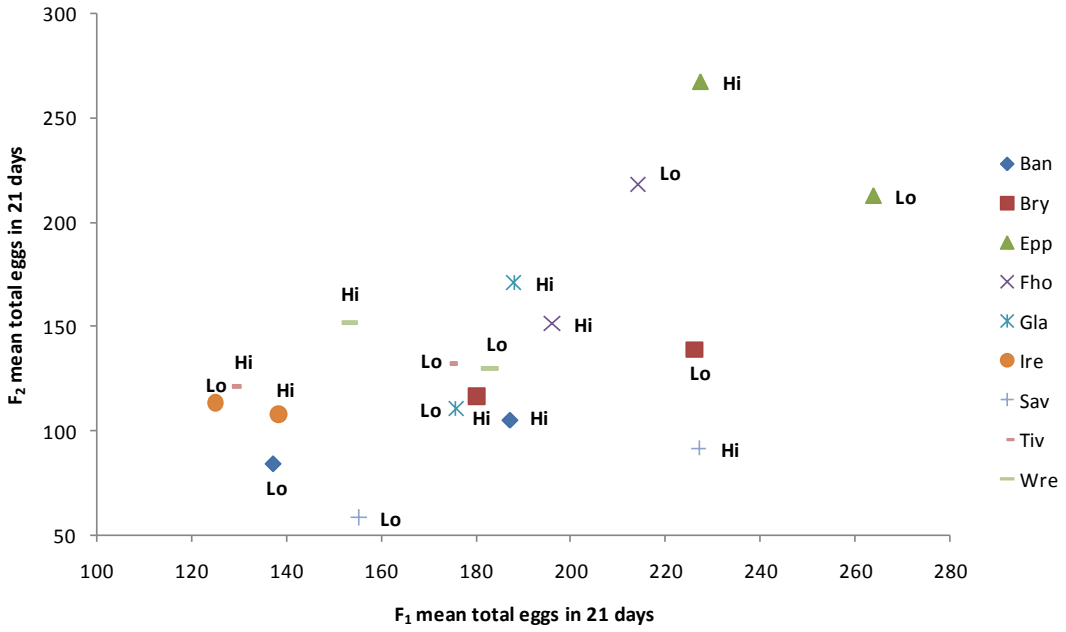


Figure 2.64. Scatter plot displaying total eggs produced in 21 days across F_1 and F_2 generations by population and high and low calcium treatment

2.4.5. Shell weight and calcium content

2.4.5.1. F_1 shell weights:

ANOVA analysis of natural log transformed F_1 shell weights (with log transformed age at cull and length as covariates) revealed a significant difference between populations, a significant effect of calcium and a significant interaction between calcium and population (See Table 2.31). The least squared mean (fitted mean) derived from the natural-log transformed weights (and accounting for covariates) for each population and calcium treatment are displayed in Figure 2.65. Significant intra-population differences in relative shell weight were found between calcium treatment and the post hoc Tukey test values are displayed below in Table 2.32.

Table 2.31. Summary ANOVA table for F_1 shell weight and calcium content.

Source	*D.F*	*F Statistic*	*P-value*	Significant
Ln Age at cull	1, 237	90.44	<0.001	Y
Ln Length	1, 237	300.59	<0.001	Y
Population	8, 237	7.29	<0.001	Y
Calcium	1, 237	114.11	<0.001	Y
Population * Calcium	8, 237	7.21	<0.001	Y

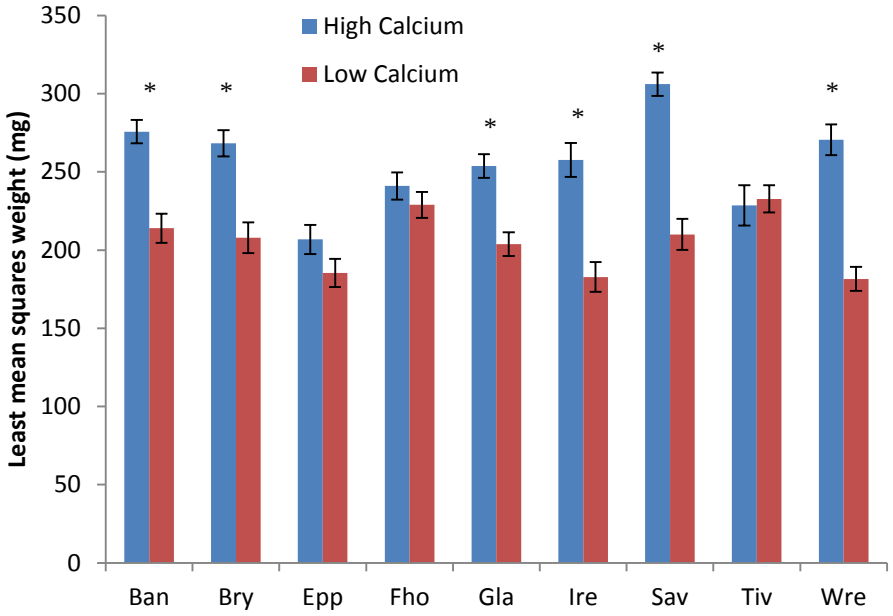


Figure 2.65. Least mean square shell weights: F_1 generation for all populations and calcium treatments. Populations marked with an asterisk display a significant difference across calcium treatment (post-hoc pairwise comparisons, P>0.05). Error bars display standard error from mean.

Table 2.32. Pairwise comparisons: Calcium treatment and factored shell weighs for F_1 generation.

Population	T-Value	P-value	Significant
Bank	**-5.154**	**<0.001**	**Y**
Brychfa	**-4.659**	**<0.001**	**Y**
Epping	-1.655	0.9728	N
Fauldhouse	-1.012	1.000	N
Glanahafren	**-4.640**	**<0.001**	**Y**
Ireland	**-5.216**	**<0.001**	**Y**
Savernake	**-7.57**	**<0.001**	**Y**
Tiverton	0.278	1.000	N
Wreake	**-7.131**	**<0.001**	**Y**

2.4.5.2. F_2 shell weights:

Due to the fact that the Bank, Fauldhouse and Savernake were reserved for further exposure to carbon black nanoparticles, comparison of relative shell weights could only be carried out for the remaining six populations from the F_2 generation. ANOVA analysis of natural log transformed F_2 shell weights (with age at cull and length as covariates) revealed a significant difference between

populations, a significant effect of calcium, and a significant interaction between calcium and population (See Table 2.33). The least squared means (fitted mean) natural-log transformed weights for each population and calcium treatment are displayed in Figure 2.66.

Table 2.33. Summary ANCOVA table for F_2 shell weight and calcium content (natural log transformed).

Source	*D.F*	*F Statistic*	*P-value*	Significant
Ln age at cull	1,162	15.34	<0.001	Y
Ln length	1,162	169.52	<0.001	Y
Population	5, 162	3.07	0.011	Y
Calcium	1, 162	21.13	<0.001	Y
Population * Calcium	5, 162	4.52	<0.001	Y

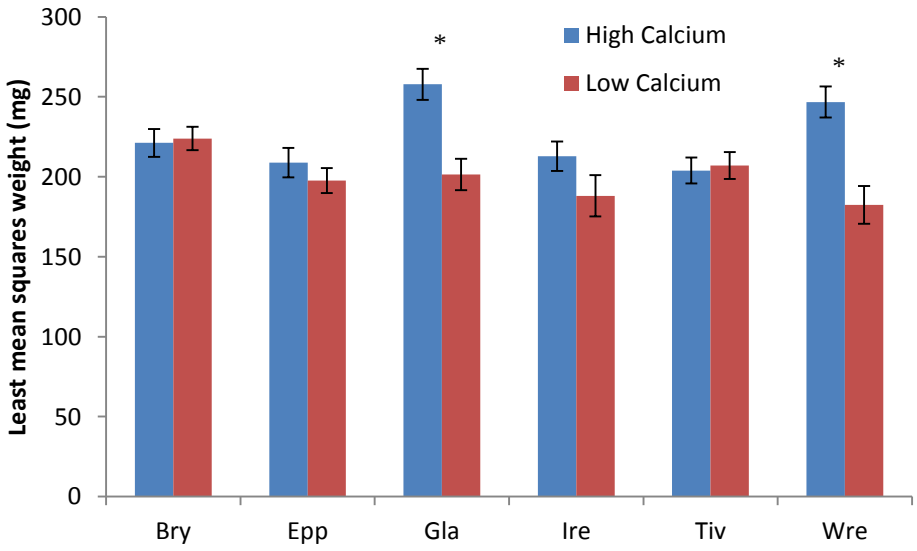


Figure 2.66. Least mean square shell weights: F_2 generation for all populations and calcium treatments. Populations marked with an asterisk display a significant difference across calcium treatment (post-hoc pairwise comparisons, P>0.05). Error bars display standard error of mean.

Significant intra-population differences in relative shell weight were found between calcium treatment and the post hoc Tukey test values are displayed in table 2.34.

Table 2.34. Pairwise comparisons: Calcium treatment and factored shell weights for F_2 generation.

Population	*T-Value*	*P-value*	Significant
Brychfa	0.232	1.000	N
Epping	-0.9486	0.9985	N
Glanahafren	**-4.090**	**0.0038**	**Y**
Ireland	-1.620	0.8994	N
Tiverton	0.276	1.000	N
Wreake	**-4.162**	**0.0029**	**Y**

2.4.5.3. Shell calcium content

To assess the relationship between shell weight and calcium content linear regression was performed. The relationship between shell weight and calcium concentration was found to be significant (DF=1, 83, F=2343.26, P<0.001) and is displayed in figure 2.67 alongside the regression equation and adjusted r-squared value.

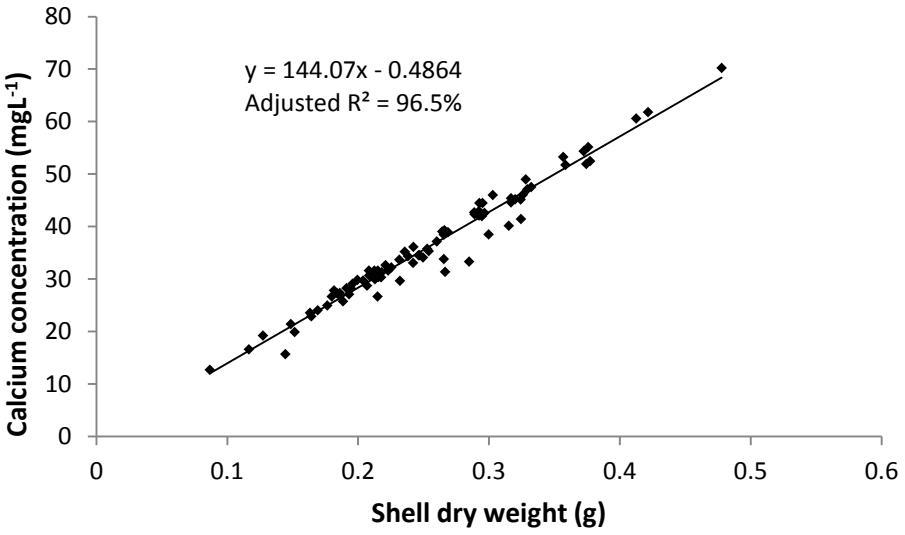


Figure 2.67. Shell weight versus calcium concentration.

In order to assess whether shell calcium content varied between populations and treatments the shell calcium data were natural log transformed and analysed via ANCOVA (with shell weight as a covariate). A significant difference in shell calcium content was found at the population level but not at

the treatment level (See table 2.35). No interaction was found between population and calcium treatment. The mean calcium to shell weight ratios for each population are displayed in Figure 2.68.

Table 2.35. Summary ANCOVA table for shell calcium content (natural log transformed).

Source	D.F	F Statistic	P-value	Significant
Shell weight	1, 66	1384.77	<0.001	Y
Population	8,66	3.17	0.004	Y
Calcium	1,66	0.01	0.906	N
Population * Calcium	8,66	1.39	0.219	N

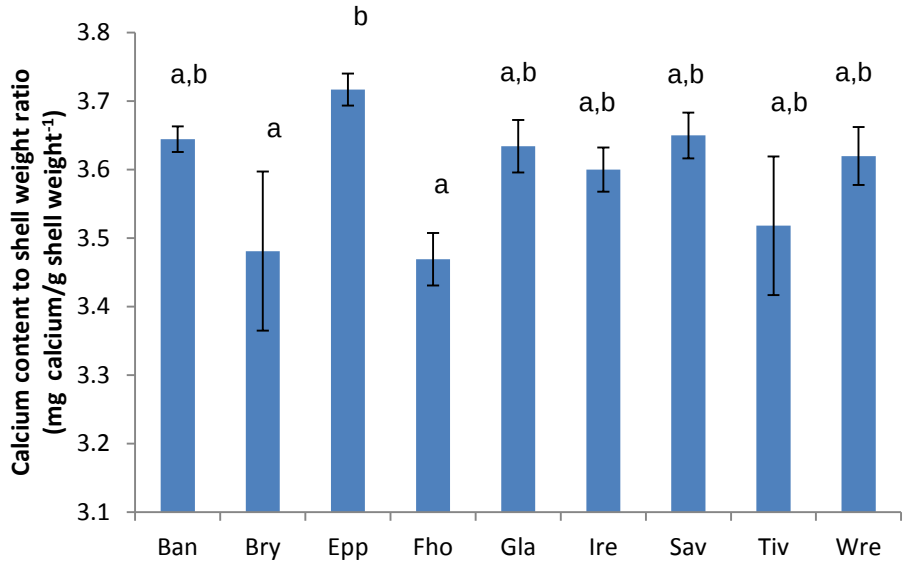


Figure 2.68. Shell calcium content to shell weight ratios: F_1 generation for all populations. Populations with the same letter have no significant difference between them (Post-hoc pairwise comparisons, P>0.05). Error bars denote standard error from mean

2.4.5.4. Inter-generational comparisons: Shell weight to length ratios

Weight to length ratios

Due to the fact that no weight to length ratio data were available for the three F_2 populations selected for nanoparticle exposure, comparison of weight to length ratios across generations was only made for the remaining six populations (Figure 2.69).

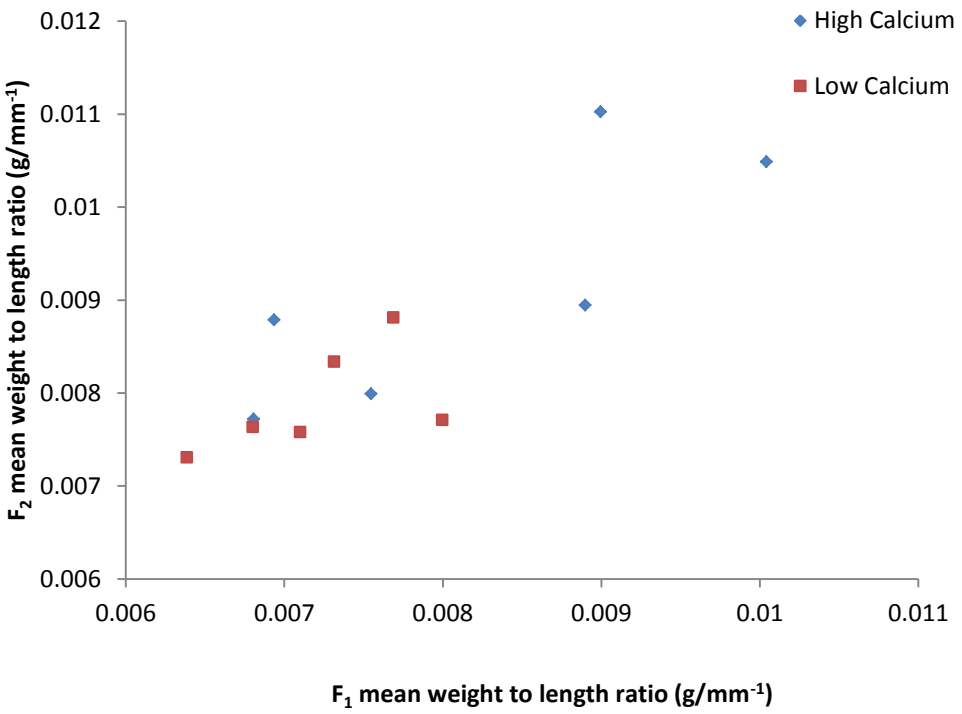


Figure 2.69. Scatter plot displaying mean weight to length ratios across F_1 and F_2 generations by high and low calcium treatment.

Shell weight to length ratios were found to significantly correlate across generations (Pearson's product-moment coefficient, n=12, r=0.829, P=0.001). From Figure 2.69 it appears that the range of values shown by different populations becomes greater in the high calcium treatment group.

2.4.6. Shell morphology

2.4.6.1. F_1 shell morphology

Raw shell measurement data were converted into ratios to account for size differences and allow direct comparison of morphological characteristics. For the F_1 generation the ratios of shell width: shell length, aperture length: shell length, aperture width: shell length, body whorl length: shell length and spire width: shell length were analysed via MANOVA across treatment and population, the summary ANOVA table being displayed in Table 2.36.

Table 2.36. Results of general MANOVA (Wilks' lambda) for F_1 shell morphology.

Parameter	Test Statistic	F-value	DF Denom	P-value	Significant
Population	0.569	3.574	1035	<0.001	Y
Calcium	0.923	3.882	237	0.002	Y
Population*Calcium	0.810	1.282	1035	0.115	N

The results in Table 2.36 indicate that there is an overall difference in morphology both at the population and treatment levels. The lack of significant interaction between the calcium and population parameter suggests that morphological responses to calcium treatment are consistent across populations.

Principal Component Analysis (PCA) of morphological data was performed and the correlation matrix and summary output with component loadings is displayed in Table 2.37.

Table 2.37. Results of PCA analysis for morphological traits, F_1 generation. SW = Shell Width, SL = shell length, AL = Aperture Length, AW = Aperture Width, BW = Body Whorl Length, SWi = Spire Width.

Axis	PC1	PC2	PC3
Eigenvalue	3.371	0.8407	0.5163
Proportion	0.674	0.168	0.103
Cumulative	0.674	0.842	0.946
Variable	**PC1**	**PC2**	**PC3**
SW:SL	0.505	-0.133	0.110
AL: SL	0.496	0.180	-0.413
AW: SL	0.355	-0.741	0.418
BW: SL	0.493	0.026	-0.472
SWi: SL	0.360	0.633	0.648

ANOVA analysis was performed on scores of principal components 1 and 2 to examine the effects of population and calcium. The results of this analysis are displayed in Table 2.38 and a plot of PC1 vs. PC2 by population is displayed in Figure 2.70. From Table 2.36 it can be seen that the PC1 parameters all display strong positive loading suggesting that for PC1 a positive increase in one morphological trait correlates with a positive increase with the others. For PC2 the strong negative loading of AW:SL is offset

against the strong positive loading of the SWi:SL variable – suggesting that as the ratio of spire width to shell length increases, the ratio of aperture width to shell length decreases.

Table 2.38. Summary ANOVA table for individual components of PCA analysis of shell morphology.

PC1 Score	D.F	F Statistic	P-value	Significant
Population	8, 241	8.25	<0.001	Y
Calcium	1, 241	1.10	0.295	N
Population * Calcium	8, 241	0.79	0.612	N
PC2 Score	**D.F**	**F Statistic**	**P-value**	**Significant**
Population	8, 241	1.97	0.051	N
Calcium	1, 241	0.29	0.593	N
Population * Calcium	8,241	2.00	0.047	Y

Pairwise comparisons for the PC1 dataset revealed that the Fauldhouse population was found to have a significantly higher PC1 values relative to the other populations (see Figure 2.70). The strong positive loadings on the PC1 variables indicate that the Fauldhouse population could be distinguished from the other populations as having shells which had higher ratio values than the other populations, the strongest loading going to shell width: shell length, aperture length: shell length and body whorl length to shell length. Thus typical shells from the F_1 Fauldhouse population would be expected to be wider, having longer apertures and body whorls relative to length, than shells from the other populations.

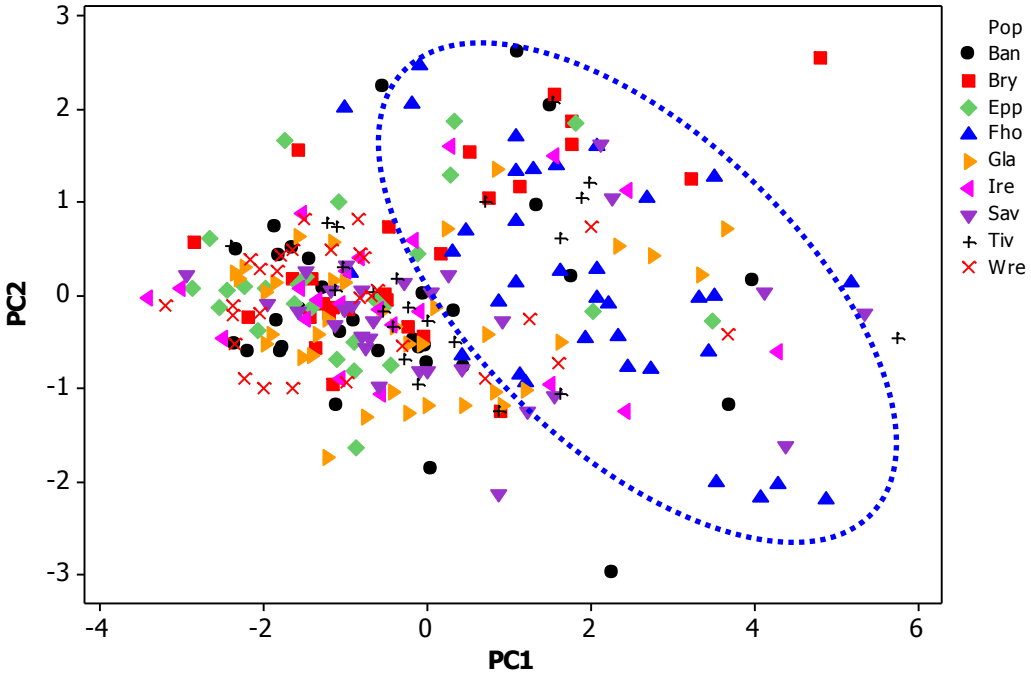


Figure 2.70. PCA analysis of shell morphology. F_1 generation. Principal component 1 vs. Principal component 2. The Fauldhouse population (blue circled) was found to be significantly different from all other populations on PC1 (ANOVA, P<0.001).

ANOVA analysis of PC2 revealed a significant interaction between calcium and population (See Table 2.38) which suggests that different populations display different morphological responses when reared in different calcium environments. It is worth noting that the vast majority (68%) of variation in the dataset can be accounted for by PC1 while only 17% can be attributed to PC2 (Table 2.37), suggesting that the most strongly varying morphological differences across populations are those described by PC1.

2.4.6.2. F_2 shell morphology

As the Savernake, Bank Well and Fauldhouse populations were retained for further study, morphological data for the F_2 generation was only gathered for the remaining six populations. Again, MANOVA analysis was performed, the results being displayed in Table 2.39.

Table 2.39. Results of general MANOVA (Wilks' lambda) for F_2 shell morphology.

Parameter	Test Statistic	F-value	DF Denom	P-value	Significant
Population	0.47316	5.286	592	<0.001	Y
Calcium	0.82920	6.550	159	<0.001	Y
Pop*Ca	0.62406	3.206	592	<0.005	Y

The results in Table 2.39 indicate that differences in morphology occur across all morphological traits examined and are detectable both at the population and treatment levels. The interaction between the calcium and population parameter suggests that morphological responses to calcium treatment are not consistent across populations, mirroring the results of the F_1 PC2 analysis.

PCA was performed on the F_2 dataset and the correlation matrix and summary output with component loadings is displayed in Table 2.40.

Table 2.40. Results of PCA analysis for morphological traits, F_2 generation. SW = Shell Width, SL = shell length, AL = Aperture Length, AW = Aperture Width, BW = Body Whorl Length, SWi = Spire Width.

Axis	PC1	PC2	PC3
Eigenvalue	3.229	1.051	0.433
Proportion	0.646	0.210	0.087
Cumulative	0.646	0.856	0.942
Variable	**PC1**	**PC2**	**PC3**
SW:SL	0.506	0.206	0.214
AL: SL	0.513	-0.201	-0.281
AW: SL	0.476	-0.056	0.715
BW: SL	0.488	-0.185	-0.577
SWi: SL	0.124	0.938	-0.178

ANOVA analysis was performed on scores on components 1 and 2 as above by population and calcium. The results of this analysis are displayed in Table 2.41 and a scatter plot of PC1 vs. PC2 by population is displayed in Figure 2.71. From Table 2.41 it can be seen that, as with the F_1 analysis, the PC1 parameters all display strong positive loading. Again a high proportion of variability in the dataset is accounted for by the first (65%) and second (21%) components. PC2 is dominated by strong positive loading of the SWi:SL

variable which is negatively and weakly correlated against AL: SL and AW: SL. and – suggesting that as the ratio of spire width to shell length increases, the ratio of aperture length and aperture width to shell length decreases.

Table 2.41. Summary ANOVA table for individual components of PCA analysis of shell morphology.

PC1 Scores	D.F	F Statistic	P-value	Significant
Population	5, 163	10.81	<0.001	Y
Calcium	1, 163	0.85	0.359	N
Population * Calcium	5, 163	10.68	<0.001	Y
PC2 Scores	**D.F**	**F Statistic**	**P-value**	**Significant**
Population	5, 163	3.86	0.002	Y
Calcium	1, 163	5.34	0.022	Y
Population * Calcium	5,163	0.78	0.565	N

Post-hoc pairwise comparisons for the PC1 scores revealed that the Epping population to be significantly different from the Brychfa, Savernake and Tiverton populations (Tukey Test, P<0.05). The Wreake population was also shown to differ from the Glanahafren, Ireland and Tiverton populations (Tukey Test, p<0.05). Both the Epping and Wreake populations had significantly lower PC1 values relative to the other populations (See Figure 2.71). The strong positive loadings on the PC1 variables indicate that the Epping and Wreake populations could be distinguished from the other populations as having shells which had lower ratio values than the other populations, the strongest loading (again as in the F_1 analysis) going to shell width: shell length, aperture length: shell length and body whorl length to shell length. Thus typical shells from the F_2 Epping and Wreake populations would be expected to be narrower, having shorter apertures and body whorls relative to length, than shells from the other populations. Significant intra-population differences in PC1 were found in the Epping population (Tukey Test, P<0.01) with shells from the high calcium treatment group having lower PC1 values, and hence a greater expression of the morphological traits described above relative to those reared in low calcium.

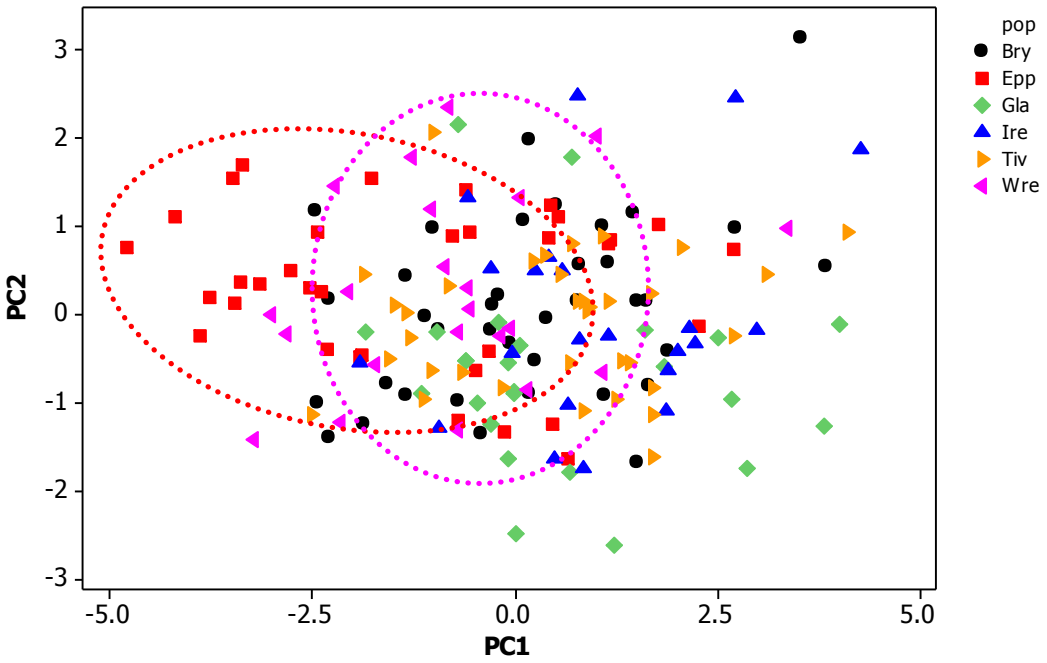


Figure 2.71. PCA analysis of shell morphology. F_2 generation. Principal component 1 vs. Principal component 2. The Epping (red, circled) population was found to significantly differ from the Glanahafren, Ireland, Tiverton and Brychfa populations, while the Wreake (purple, circled) population was found to significantly differ from the Glanahafren, Ireland and Wreake populations in PC1 (ANOVA, P<0.001).

The interaction plot between calcium and population for PC1 is displayed in Figure 2.72. It is of interest to note that the differences between traits become more pronounced in the high calcium treatment groups but their nature and magnitude are not conserved throughout – as indicated by the significant interaction term in Table 2.41.

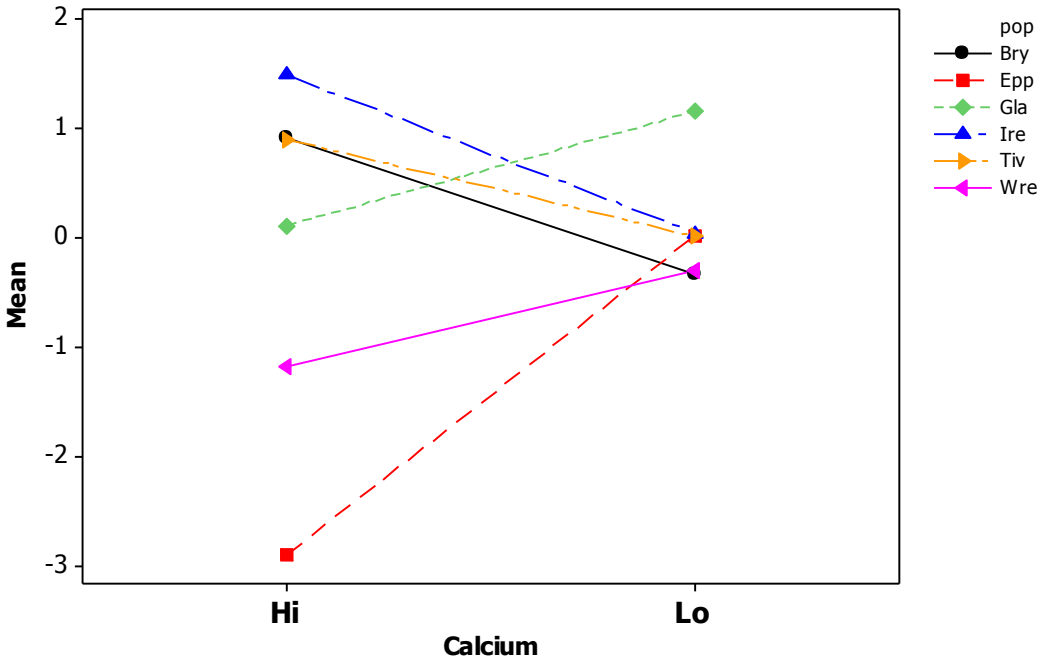


Figure 2.72. Interaction plot displaying mean PC1 scores across calcium treatment. Only the Epping population was found to display significant intra-population differences in PC1 response to calcium treatment.

Post-hoc analysis of the PC2 values revealed significant differences at the population level between the Glanahafren population and the Epping, Wreake and Brychfa populations (Tukey test, $P<0.05$). The mean PC2 scores are displayed in Figure 2.73. As the greatest loading in PC2 was assigned to SWi:SL (see Table 2.40) it would suggest that the overall morphological differences between populations are mostly driven by this trait and that shells from individuals from the Glanahafren population, with PC2 values lower than other populations, are more likely to differ from the other populations by having thinner spires relative to shell lengths.

The PC2 analysis also detected an overall difference in PC2 between calcium treatments. Mean PC2 scores were 0.164±S.E 0.1.11in the high calcium treatment group as opposed to -0.174±S.E. 0.105 in the low calcium group. The lack of interaction between calcium and population (see Table 2.41) would suggest that the nature of morphological differences in response to

calcium treatment is consistent across populations whereby, in general, snails reared in high calcium tend to be characterised as having a thicker spires relative to shell lengths than their low calcium counterparts.

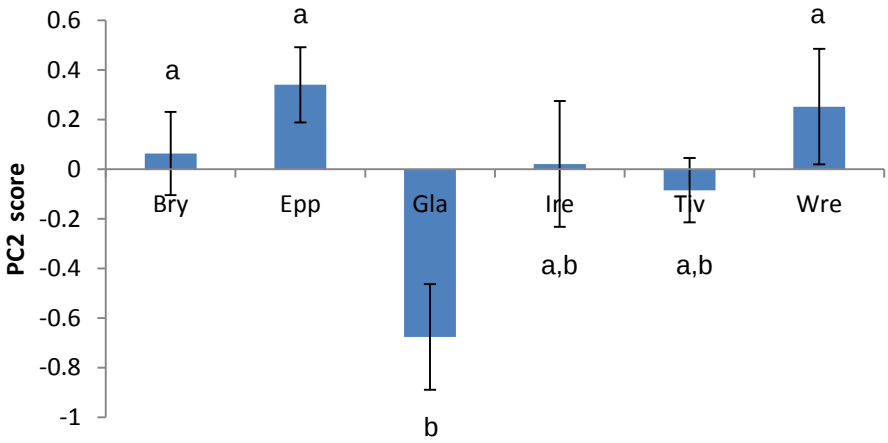


Figure 2.73. Mean PC2 scores across for each population. The Glanahafren population displayed significant differences from the Wreake, Brychfa and Epping populations (letters denote populations with no significant differences between means). Error bars denote standard error of mean.

2.4.6.3. Shell morphology: Inter-generational comparisons

Shell Morphology

Due to the removal of individuals to be used in the nanoparticle exposure in the F_2 generation, morphological comparison across the F_1 and F_2 generations could only be made for six populations – with the Fauldhouse, Savernake and Bank Well populations being removed.

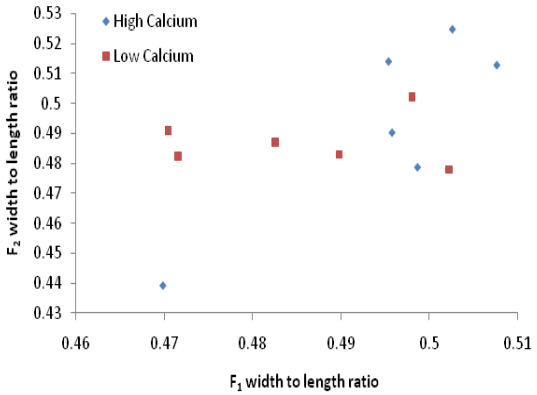
Five shell morphology traits were compared across generations: shell width: shell length, aperture length: shell length, aperture width: shell length, body whorl length: shell length and spire width: shell length. Figure 2.74 displays all traits analysed across generations. Shell width: shell length, aperture width: shell length, and body whorl length: shell length ratios were all found to display a significant correlation across the F_1 and F_2 generations (Pearson's product-moment coefficient, n=12 for all comparisons: r=0.603, p=0.038, r=0.780, p=0.003 and r=0.601, p=0.039 respectively). Morphological traits were also analysed by calcium treatment alone to assess whether calcium

regime affected the heritability of traits across generations and the results are displayed in Table 2.42.

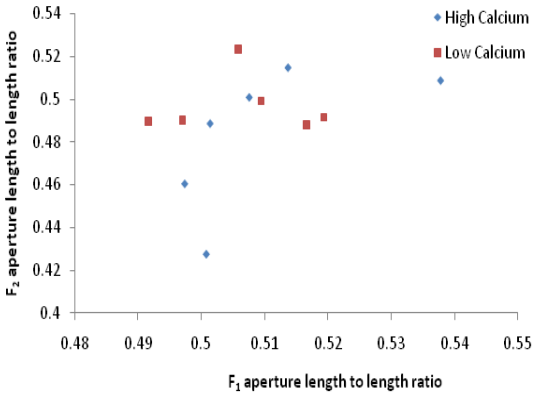
Table 2.42. Correlation between morphological traits across F_1 and F_2 generation separated by calcium treatment (Pearson's product-moment coefficient).

Variable	*Low Calcium*				High Calcium			
	n	**R**	**P**	**Sig**	**n**	**R**	**p**	**Sig**
SW:SL	6	0.047	0.930	N	6	0.870	0.024	Y
AL: SL	6	0.010	0.986	N	6	0.616	0.193	N
AW: SL	6	0.884	0.019	Y	6	0.785	0.064	N
BW: SL	6	0.307	0.554	N	6	0.750	0.086	N
SWi: SL	6	-0.280	0.592	N	6	0.148	0.782	N

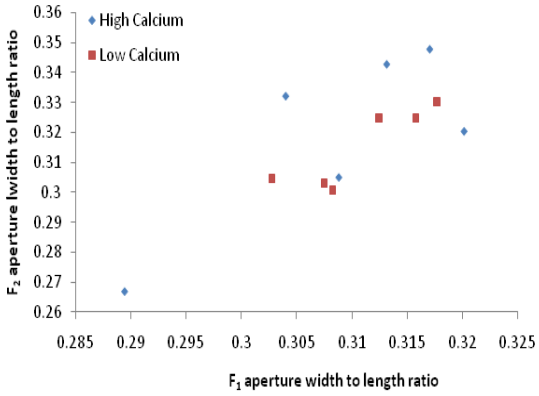
i. Shell width to length ratio.



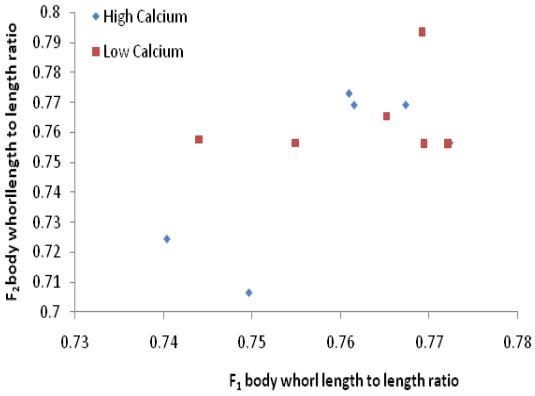
ii. Aperture length to shell length ratio.



iii. Aperture width to shell length ratio



iv. Body whorl length to shell length ratio.



V. Spire width to shell length ratio.

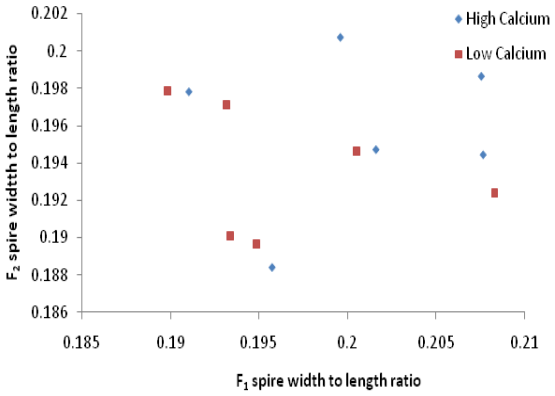


Figure 2.74. Scatter plots (i-v) displaying F_1 and F_2 shell morphological trait comparisons for six populations (Bry, Epp, Gla, Ire, Tiv, Wre) by high and low calcium treatment.

Multivariate morphology comparisons

In order to be able to compare multivariate patterns of morphological differences between generations, PCA was performed on the F_1 dataset with the three populations not present in the F_2 generation removed. The results of the analysis are displayed in Table 2.43.

Table 2.43. Comparison of results of PCA analysis for morphological traits, F_1 & F_2 generations for six populations (Bry, Epp, Gla, Ire, Tiv, Wre). SW = Shell Width, SL = shell length, AL = Aperture Length, AW = Aperture Width, BW = Body Whorl.

Parameter	***F_1 PC1***	***F_1 PC2***	***F_2 PC1***	**F_2 PC2**
Eigenvalue	3.2720	0.8274	3.2288	1.0510
Proportion	0.654	0.165	0.646	0.210
Cumulative	0.654	0.820	0.646	0.856
Variable	**PC1**	**PC2**	**PC1**	**PC2**
SW:SL	0.506	-0.096	0.506	0.206
AL: SL	0.491	0.234	0.513	-0.201
AW: SL	0.325	-0.860	0.476	-0.056
BW: SL	0.488	0.082	0.488	-0.185
SWi: SL	0.398	0.436	0.124	0.938

A comparable amount of variation in the dataset can be accounted for by the first two axes in both F_1 and F_2 generations (>80%). From Table 2.43 it is apparent that PC1 values are positively correlated for both the F_1 and F_2 generations. In both generations the three strongest traits are the same (SW:SL, AL:SL and BW:SL) and in the case of SW:SL and BW:SL the loadings are identical, suggesting that differences in shell morphology detected by PC1 are conserved across generations. For PC2 the strong negative loading of AW:SL in the F_1 generation is not apparent in the F_2 generation, with SWi:SL being the strongest contribution instead. The strongest similarity in PC2 values comes from the positively loaded SWi:SL ratio, which displays the strongest positive loading in both generations.

2.5. Discussion

2.5.1. Site and study environmental conditions

Environmental calcium was found to exceed 25 mgL^{-1} in the Wreake, Ireland, Bank Well and Epping sites, with highest values in excess of 70mgL^{-1} being found in the Wreake and Ireland sites. Intermediate calcium levels (>10 mgL^{-1}, <25 mgL^{-1}) were found at the Tiverton, Glanahafren and Brychfa sites, and lowest at the Savernake and Fauldhouse sites (<10 mgL^{-1}). Boycott (1936) described *L. stagnalis* as a calciphile species, requiring environmental calcium concentrations of > 20mgL^{-1}. From the site calcium data it is clear that *L. stagnalis* is capable of tolerating environmental calcium as low as 2.11mgL^{-1} as recorded at the Savernake site and it has been suggested by Young (1975) that snails found at low calcium sites supplement calcium requirements by diet.

Freshwater lake ecosystems can be defined by their nutrient concentrations (Moss, 1998). Typical trophic groupings alongside the corresponding nutrient and photosynthetic pigment concentrations (chlorophyll a) as defined by Nürnberg (1996) are displayed below in Table 2.44.

Table 2.44. Trophic states and their characteristic nutrient and chlorophyll *a* (Chl *a*). TN = Total nitrogen, TP = Total phosphorus. Modified from Nürnberg (1996).

Trophic state	TN (µg L-1)	TP (µg L-1)	Chl *a* (µg L-1)
Oligothrophic	< 350	< 10	< 3.5
Mesotrophic	350-650	10-30	3.5-9
Eutrophic	650-1200	30-100	9-25
Hypertrophic	> 1200	> 100	> 25

The site TON concentrations displayed in Figure 2.20 indicate that TON concentrations are spread across the full range of trophic state classification: from hypertrophic (Wreake), to eutrophic (Glanahafren and Brychfa), to mesotrophic (Bank), with the remaining sites falling into the oligotrophic classification. Site phosphate was found to range from eutrophic levels (Savernake and Bank), to mesotrophic (Tiv and Ireland), to oligotrophic for the

remaining sites. In summary nutrient levels were shown to vary by an order of magnitude across the sites, with no consistent pattern appearing with respect to the different nutrients.

Despite attempts to choose sites that were thought to be minimally impacted on the basis of existing data, there were significant variations in nutrient levels which are likely to be linked to anthropogenic inputs. These could possibly have influenced the patterns of variation evident in the laboratory experiment results through effects on life history traits, but it was impossible to explore these further without significant additional experimentation that was beyond the scope of the current work.

Experimental environmental conditions

Temperature was maintained at a consistent level of around 17±1°C throughout the experiment, with diurnal variation most likely attributable to heating by the lighting system.

Mean site TON levels were shown to climb rapidly in all systems to approximately 3mgL^{-1} during the first year of study. Thereafter TON values displayed considerable variation between systems with A and D displaying gradual increase towards maximal values of 4.49mgL^{-1} and 4.75mgL^{-1} respectively, and systems B and C displaying a steady decline towards approximately 2mgL^{-1} by the end of the experiment. Clearly the TON concentrations in all systems were found to exceed the levels described in Table 2.44 and all systems can be considered to have become hypertrophic with respect to TON by the end of the study.

A similar trend was displayed in phosphate values, whereby a steady increase in systems A, C and D was noted throughout the study. System B did not show the same trend as the others, displaying consistently low phosphate concentrations throughout the experiment. It is likely that the lower phosphate levels observed in system B were attributable to uptake by algae. Algal contamination was noted in all systems throughout the study but appeared to be far more established in system B. The highest phosphate value of

1.06mgL^{-1} was found in system A towards the end of the study. Again it would appear that, with the exception of system B, hypertrophic levels of enrichment were found in all other systems by the end of the study.

The observed increase in TON and phosphate values over time in the systems was most likely derived either directly from the iceberg lettuce or from snail excreta. Despite the relatively high nutrient levels reported oxygen saturation levels remained close to 100% (Table 2.4).

2.5.2. Life history trait variation across population and calcium treatments

This section will aim to summarise and interpret the observed differences in life history response between different populations and across calcium treatments, and endeavour to place these findings in the context of current life history theory. This section will focus on individual traits, highlighting where salient patterns occur at the population and treatment levels. A full analysis of interactions between traits (trade-offs) will be covered in Chapter 3. Due to the extent of the results being discussed, it is intended to initially briefly summarise the main patterns shown for each trait considered, before going on to discuss the significance of the observed patterns subsequently. A summary of the main findings of the life history work described in the results section is displayed in Table 2.45.

Table 2.45. Summary of general findings of life history studies presented in chapter 2. Statistically significant results are denoted by (*), P<0.05, (**), P<0.01, (***), P<0.001, and (–), denotes non-significant result. N/A denotes not applicable/not performed.

Trait/Character	*F_1 Generation*			*F_2 Generation*			*Evidence of Heritability*
	Population	**Calcium**	**Pop * Ca**	**Population**	**Calcium**	**Pop * Ca**	
Growth:							
Initial growth	**	-	*	***	***	***	N/A
Isolated Growth:							
Parameter *a*	**	-	*	***	***	***	-
Parameter *b*	**	***	***	**	-	**	-
Parameter *c*	-	**	**	***	***	-	-
Survivorship:							
Egg survivorship	*	-	**	***	-	-	-
Initial survivorship	*	-	N/A	***	-	-	-
Individual survivorship	***	-	**	***	***	**	N/A
Reproduction:							
Age at 1st reproduction	***	***	***	***	**	-	-
Size at 1st reproduction	***	***	**	***	-	**	* R^2 = 0.34
Total number of egg masses	-	-	-	***	-	-	* R^2= 0.24
Total number of eggs	*	-	-	***	-	-	** R^2= 0.39
Eggs per mass	**	-	*	***	**	-	-
Shell composition:							
Shell weight	***	***	***	*	***	***	** R^2= 0.69
Shell calcium content	**	-	-	N/A	N/A	N/A	N/A
Shell morphology:†	***	**	-	***	***	**	N/A
Morphology PC1	***	-	-	***	-	***	Yes (PC loadings and
Morphology PC2	-	-	*	**	*	-	No ranks consistent)
Shell width: Shell Length	N/A	N/A	N/A	N/A	N/A	N/A	* R^2= 0.36
Aperture width: Shell length	N/A	N/A	N/A	N/A	N/A	N/A	** R^2= 0.61
Body whorl length: Shell length	N/A	N/A	N/A	N/A	N/A	N/A	* R^2= 0.36

†Derived from general MANOVA of all morphological data.

Hatchling groups

Hatchling group growth was shown to differ across populations in both the F_1 and F_2 generations, although the way it was measured also differed between generations. In the F_1 generation consistently high growth rates were displayed by the Bank, Brychfa and Fauldhouse populations in both high and low calcium treatments, while the Epping and Wreake populations displayed the lowest growth. Greatest differences between growth rates across calcium regime were found in the Savernake and Ireland populations (greater growth in the high calcium treatment) and the Tiverton population (greatest growth in the low calcium treatment), however none of these trends were found to be significant at the intra-population level.

Growth in the F_2 generation hatchling groups (which was calculated by length to age ratio and is thus not directly comparable with the F_1 data) tended to be faster in the low calcium treatments for Bank, Brychfa, Epping, Fauldhouse and Ireland populations, however this relationship was only found to be significant in the Fauldhouse population and the relationship was not found to be conserved throughout all populations. The Fauldhouse and Ireland populations displayed the greatest differences in growth response across calcium treatment, with highest growth being recorded in the low calcium treatment groups. The highest and lowest overall growth was recorded in the Fauldhouse population while the Bank, Brychfa and Epping populations displayed marginally higher growth than the others.

There was some consistency in the relative growth rates between generations, but not for all populations. The observed changes in growth rate in response to calcium treatment in the different generations would tend to suggest that early growth rates are indicative of a response to underlying genetic variation, rather than being a mix of genetic and environmental factors, where a common response to environmental differences would be expected to become manifest after two generations (Stearns, 1992). This is in contrast to Byrne *et al* (1989) who found that most of the variation in growth rate was non-genetic and attributable to environmental influences.

Growth parameter analysis: F_1 generation

As would be expected, differences in response were more readily detected at the population level and significant intra-population difference in growth rates (parameter *c*) were only found in the Brychfa population. The F_1 parameter *a* (asymptotic size) and parameter *c* values revealed a similar trend in the order of response across calcium treatment to the F_1 x10 replicate data. With respect to growth across calcium treatment, all populations with the exception of the Bank, Brychfa and Epping populations were shown to mirror the order of response displayed in the F_1 x10 replicates, e.g. Ireland and Savernake show higher growth response (both in terms of asymptotic size and growth rate) in high calcium treatment while Tiverton shows the reverse trend. Parameter *b* (predicted initial size) was shown to vary significantly for the Brychfa, Ireland and Savernake populations, with significantly larger individuals being found at t=0 in the low calcium treatments. Parameter *b* values tended to be in the reverse order of parameter *a* and *c* values suggesting that large initial size resulted in relatively lower growth rates and final size across calcium treatments. Parameter *b* values did not display as closely similar trends to the x10 replicate data as parameters *a* and *c*.

It would appear that differences in the initial F_1 growth rates across calcium treatments were carried through to differences in overall growth rates and final size of adult individuals, although differences in overall growth at the population level would appear to vary at different growth stages (e.g. Epping displays low growth relative to other populations at the x10 replicate stage, then higher growth in the linear phase (parameter *c*)). The results suggest that population differences in growth are readily detectable in the F_1 generation, while the effects of calcium regime appear to be relatively subtle. While they are not consistent across all populations, there appears to be an overall trend towards higher growth at high calcium levels. With respect to the fitted Gompertz growth curves, this effect is most clearly seen in the Brychfa, Ireland and Savernake populations.

F_2 generation

As with the F_1 generation, population differences were readily detected in all growth parameters analysed but the detection of intra-population differences proved to be more elusive. Further, the differences observed in the F_1 generation were not conserved across generations and in the F_2 generation different populations showed significant differences in parameters when compared with those in the F_1 generation. For example, the Epping, Glanahafren and Wreake populations were shown to tend toward significantly larger asymptotic size in the high calcium treatment while the Fauldhouse population was shown to display larger individuals in the high calcium treatment at t=0. Some mild consistencies between generations were apparent such as the Epping population displaying amongst the highest growth rates but in general the F_1 growth analysis bore minimal similarity to the F_2 analysis.

Of significance in the F_2 study was that overall, conserved higher growth rates were found across all populations in the high calcium treatment groups. This is suggestive that the high variability in environmental response displayed by the F_1 generation was due to plasticity influenced by environmental variation which has been 'bred out' in the F_2 generation allowing a conserved response to calcium treatment to be observed.

Generational comparisons

Unsurprisingly, no relationship between F_1 and F_2 growth parameter terms was found when correlations were performed on each term. When analysed separately the low calcium asymptotic size data were found to approach significance ($p=0.063$), suggesting a genetic component in relation to absolute shell size. That this relationship is more readily detectable in the low calcium treatments would be expected as inter population differences would be expected to become more manifest when resources are limited (Lam, 1999).

Overall combined growth parameter terms were found to differ significantly between the F_1 and F_2 generation. Parameter *a* was shown to be significantly lower by approximately 3mm in the F_2 generation, indicating that the F_2

generation attained a significantly lower asymptotic size than the F_1 generation. The reverse trend was apparent with respect to growth rate (parameter *c*), where the F_2 generation was shown to display significantly faster growth than the F_1 generation. The observed effects could be a result of inbreeding of the F_1 generation (Charlesworth and Charlesworth, 1987).

Survival

Lifetime survivorship can be classified into the three distinct curves, with age on the x axis and survivorship on the y axis, as described by Deevey (1947). Type I depicts a convex curve with high juvenile and adult survivorship, with highest mortality occurring towards the end of the natural lifespan (as displayed by humans). By contrast type III survivorship, as displayed by many invertebrates and marine fish species, is characterised by a concave curve depicting high juvenile mortality, with surviving individuals displaying high subsequent survivorship. Type II survivorship represents the straight line intermediate curve, where mortality is held constant throughout the given organism's lifespan and is typical of the strategy employed by seed bank derived plants (Begon et al., 2006). When studying survivorship at the population level the type of survivorship strategy employed will influence where differences in survival are likely to be detected. For example, when an organism displays a type II type survivorship curve, inter population differences in survivorship may be expected to appear at any time throughout an organism's life, while when a type I or type III curve is displayed, differences in survivorship are likely to be more pronounced at late or early life stages respectively. A field study by Brown (1985) estimated a 2% survivorship to maturity in *L. stagnalis* with this statistic being driven by high juvenile mortality rates. In this study, survivorship rates of eggs was of comparable magnitude to the range of survival values across the remainder of the life cycle up to adult maturity and reproduction. This corresponds to the type III convex survivorship curve as described by Deevey (1947), with highest mortality occurring in early life stages relative to later ones.

Hatchling groups

Average mortality in the F_1 hatchling groups was found to be around 10%. Significant inter population differences in mortality were observed, but these differences did not appear to be strongly affected by environmental calcium Lowest survivorship was found in the Epping population which displayed approximately 10% higher mortality than the other populations. This pattern was conserved and much more pronounced in the F_2 x10 replicate data, with the Epping population displaying approximately 40% lower survivorship relative to other populations. Calcium treatment was again found to not strongly affect survivorship. Overall survivorship in the x10 replicate stage was found to be significantly lower in the F_2 group. In general the F_2 population was shown to display mortality of approximately 30%, which was around 20% higher than that displayed by the F_1 generation. Some populations appeared to display greater inter generational differences than others with greatest reductions in survivorship being recorded in the Epping (~40%), Brychfa and Wreake (~30%) populations while the Savernake population displayed virtually no difference in survivorship across generations. The observed general increase in mortality across generations is likely the effect of inbreeding due to selfing (Charlesworth and Charlesworth, 1987) and suggests that a strong effect of inbreeding on juvenile survival is readily detectable within a generation (Charlesworth and Charlesworth, 1987, Coutellec and Lagadic, 2006). That this effect is due to inbreeding and is genetic in origin is corroborated by the fact that a similar response across generations was recorded in other life history traits such asymptotic shell growth and reproductive output (discussed later), where the F_2 generation was shown to grow to a significantly smaller size overall than the F_1 generation.

Individual survival

A conserved response across calcium treatment did not become apparent until the F_2 generation, whereby approximately 10% lower survival was detected in the low calcium treatments. Significant intra population differences in mortality were only detected for the Tiverton population in the F_1 generation and in the Bank population in the F_2 generation. The detection of

a conserved population level response to calcium in only the F_2 generation is suggestive that mortality response to calcium concentration is subtle and may be masked by other environmental influences only becoming apparent when such influences are removed. Further, increased inbreeding implicit in the F_2 generation and the disadvantages that this may infer to other life history traits (growth and juvenile survival as described above) may also result in differences in response to other stressors (in this case calcium) becoming more apparent (Coutellec and Lagadic, 2006). In general the high degree of inter population variation, with no distinct pattern being apparent across generations, would appear to suggest that environmental effects other than calcium may be more important in directly influencing the observed differences in mortality. Whilst mortality is clearly a highly variable trait, it does not appear to be significantly affected by calcium availability at the levels used.

Differences in early survival are not always reflected through to adult survival (Begon et al., 2006). For example the Epping population was shown to display high adult survival in both the F_1 and F_2 generations despite high juvenile mortality. By contrast the Bank population in the F_2 generation showed marked differences in adult survival across calcium treatment but relatively high survival at the x10 replicate stage. Such differential survival between early and late life stages may be part of a trade-off within the overall life history strategies of the given populations linked to variation in reproductive strategy (Stearns 1976).

Reproduction: Age and size at first reproduction

The age at first reproduction serves as a milestone in any given organisms' life history (Stearns, 1992). The onset of maturity and first reproduction marks a shift in the channelling of resources away from purely somatic growth towards a state where somatic growth and gametogenesis compete for the finite resources available (Harshman and Zera, 2007). The cost of reproduction (CR) may differ across habitats, being affected by many extrinsic biotic and abiotic factors ranging from predation (Lewis, 2001, Reznick, 1983, Reznick et al., 1990) to geographical clines (Cardoso and Defeo, 2004) and

may drive a shift in the age at first reproduction. When the costs of reproduction are high (in the face of intense competition (Cluttonbrock, 1984) or size specific predation (Reznick, 1983)) a shift towards delayed maturity (typically associated with larger size) and a reduction in reproductive allocation may occur. In contrast, where the costs of reproduction are low (where indiscriminate size and age independent mortality occurs, or in the absence of competition) a shift towards early maturity, at a smaller size, with an increase in reproductive investment is to be expected (Begon et al., 2006).

Stearns (1992) suggests that such observed patterns of variation on age and size at maturity result from a fundamental trade-off between juvenile growth and survival and subsequent adult reproduction. Factors which influence juvenile survival and growth such as food and nutrient availability (such as environmental calcium) or predation, are therefore likely to elicit an effect on age at first reproduction.

Age at first reproduction

Overall age at first reproduction was found to be higher in the F_2 generation, with the study populations as a whole taking approximately 80 days longer to reach first reproduction than the F_1 generation. Such retardation of first reproduction is in keeping with the effects associated with inbreeding as described earlier in this chapter (Charlesworth and Charlesworth, 1987, Coutellec-Vreto et al., 1998). While some populations (as indicated by significant interaction term) in the F_1 generation displayed the reverse trend, the dominant trend was for snails to take longer to reach reproductive age in the low calcium treatments. This was followed by a conserved response across populations in the F_2 generation with low calcium treatments taking longer to reproduce than their high calcium counterparts. In the F_1 study the Savernake, Ireland and Brychfa populations were shown to display significant intra population difference in age at first reproduction, taking longer to reproduce in the low calcium treatments, a trend which is mirrored by these populations displaying lower growth rates (parameter *c*, only Brychfa significant) in low calcium treatments (Figure 2.32). This would suggest a trade-off between growth rate and age at reproduction and would appear to

be sensitive to calcium availability. This general trend is carried on into the F_2 generation where the lower growth rates found conservatively in the low calcium treatment group (Figure 2.36) appear to correspond with an overall trend towards later reproduction in this group (Figure 2.51).

The consistency across generations would support the view that a trade-off between growth and age at first reproduction is genetic in origin and subject to environmental influence, although the lack of strong significance in response across calcium treatments would suggest that this effect is only in part derived from environmental calcium concentration and that other environmental factors may contribute. Although generational comparisons failed to find a significant inter generational correlation between F_1 and F_2 age at first reproduction, further support that this trait may be under genetic control (at least in some populations) comes from the fact that the Tiverton population was shown to consistently reproduce first across generations (in both high and low calcium treatments), while support for an interaction with environmental calcium comes from the fact that the Fauldhouse population was shown to consistently take the longest to reproduce in the low calcium treatment across generations. Full consideration of trade-offs and interactions between traits will be covered in Chapter 3.

Size at first reproduction

With the exception of the Wreake population the F_1 snails in the low calcium group were larger at first reproduction that those in the high calcium group. Significant intra population differences were found in the Brychfa and Fauldhouse populations with snails being larger in the low calcium treatment at onset of reproduction. This would suggest that in the face of limiting resources in the form of calcium availability that reproduction would be delayed in favour of reaching a particular size. Different species may use different 'rules' as to when to reproduce (age versus size) but in freshwater molluscs, reproductive output is more closely linked to size than age (Calow 1978, 1983, Dillon 2000) suggesting that size is more critical. However, the same trend was not observed in the F_2 generation, where calcium was found to have no effect on size at first reproduction and intra population differences

were not readily detectable. Unlike other traits discussed thus far, size at first reproduction appeared to be strongly conserved between the F_1 and F_2 generations, with a significant correlation being recorded in this trait. When analysed separately a significant correlation was found only in the low calcium treatment group, suggesting that genetic differences are more readily detected across generations when environmental stress is greater (Lam, 1999). It would thus appear that size at first reproduction is under stronger genetic control and displays less of a response to inbreeding than other traits analysed thus far, possibly as this is more closely related to fitness (Stearns 1989).

Population differences in size at first reproduction did not show any consistency with age at first reproduction or other growth parameters across either generation. For example, the Fauldhouse population was shown to be the largest at first reproduction across both generations but middle ranked in age at first reproduction in the F_1 generation and highest ranked in the F_2 generation while the Ireland population was shown to be small at first reproduction but middle ranked in terms of age. This may be the result of differential investment in reproduction as the adult stage is approached and will be discussed later.

The Fauldhouse population was the most northerly of all the study populations. As mentioned above, this population was shown to reach the largest size at first reproduction in both generations. This of interest as it is consistent with other studies that report increases in body size with latitude in other species (Cardoso and Defeo, 2004, Lardies and Bozinovic, 2008). Reproductive output in freshwater gastropods is typically a function of adult size (McMahon, 1983) and it is possible that the larger size at first reproduction displayed in this population reflects a greater investment in reproduction due to increased environmental stressors associated with higher latitudes. Other studies report an increase in offspring size and reduction in number associated with high latitude populations, whereby larger offspring are more likely to survive in harsher abiotic environments (Hassall et al., 2006, Olsson and Agren, 2002, Yampolsky and Scheiner, 1996). In *L. stagnalis*,

and freshwater molluscs in general, increased investment in reproduction associated with larger adult size is more likely to take the form of an increase in egg number rather than size, for lineage specific reasons discussed below.

Reproduction: Number and size of offspring

The above population differences in resource allocation towards pre reproductive growth would be expected to influence the amount of energy subsequently available for reproduction in the form of a trade-off (Stearns, 1989b). *L. stagnalis* display a positive correlation between adult fecundity and parent size, which coupled with this trade-off between growth and reproduction, could select for different life history strategies whereby smaller snails reproducing earlier would be expected to have lower fecundities than larger snails reproducing later (Calow, 1983, Dillon, 2000). Such differences in fecundities could result from trade-offs between not only the number and size of eggs produced but also between the frequency of egg masses laid. The typically negative relationship between egg size and number is well documented in the ecological literature and has been readily observed across many taxonomic groups (Begon et al., 2006, Stearns, 1992). However in freshwater molluscs this relationship has been shown to sometimes be positively related to adult size and does not display strong intra specific variation with the implied trade-off between egg size and number as it does in other groups (Dillon, 2000). Due to this and time constraints the size of eggs was not measured in this study, but it is possible that population differences in egg size were present and would glean further insight into reproductive strategies across the study populations and should require further investigation. The discussion below will therefore only focus on the number of egg masses, the number of eggs and the number of eggs per mass.

The number of egg masses produced in 21 days did not significantly differ at the population or treatment levels in the F_1 generation (although significance was approached at the population level). Differences at the population level became more pronounced and were detected by the F_2 generation.

The Tiverton, Ireland and Savernake populations consistently produced the least egg masses across generations while the Brychfa and Glanahafren populations produced the most egg masses. When the number of egg masses produced and the size of individuals at reproduction was compared there appeared to be a tentative relationship between reproductive output and size, whereby the Ireland, Savernake and Tiverton populations tended to be smaller at first reproduction and produced fewer egg masses. Significantly more egg masses were produced overall in the F_2 generation which may be suggestive of a compensatory response to counteract the effects of inbreeding on survivorship (Charlesworth and Charlesworth, 1987).

When compared across generations a significant correlation between the F_1 and F_2 generations was observed. This would suggest that inter-population differences between the number of egg masses produced is strongly genetically determined. The lack of intra population variation/plasticity (and therefore genetic variation) in egg mass production in response to environmental calcium would further support this view. Calow (1983) suggests that the production of egg capsule represents a significant investment of resources and that variation in the number of eggs per mass is likely to be more readily detectable due the high costs associated with egg mass production.

The total number of eggs produced in 21 days more closely mirrored size at first reproduction than the total number of egg masses produced, and again was largely conserved across generations. The Savernake, Ireland and Bank populations consistently ranked among the lowest egg producers, while the Epping and Fauldhouse consistently ranked as the two highest between the F_1 and F_2 generations. Significantly fewer eggs overall were laid in the F_2 generation. As well as producing fewer eggs overall, the F_1 Ireland and Bank populations were also shown to produce fewer eggs per mass while a similar patterns emerged for the highest egg producers (Fauldhouse and Epping). The same trend was evident in the F_2 generation, with the Savernake population producing the lowest total number of eggs and being second lowest in terms of eggs per mass. The Epping and Fauldhouse populations

again were among the highest total egg producers and produced more eggs per mass.

The number of eggs per mass displayed a significant interaction between population and calcium in the F_1 generation, with the Fauldhouse and Savernake populations clearly displaying a trend towards fewer eggs per mass in the low calcium treatments. A conserved response, whereby fewer eggs per mass overall were laid in the low calcium treatments, became evident by the F_2 generation. No significant correlation in eggs per mass was detected between generations and significantly fewer eggs per mass were laid in the F_2 generation.

The above differences between populations indicate that pronounced differences in reproductive strategy in terms of total eggs produced and number of eggs per mass exist at the population level for *L. stagnalis*. Population differences in total egg numbers appear to be conserved across generations, suggesting that total reproductive output may be genetically fixed and may be influenced by other aspects of *L. stagnalis* life history, such as survivorship at different life stages. Further, different populations appear to select somewhere between a large number of egg masses with fewer eggs or fewer egg masses, with more eggs. While both strategies can theoretically result in the same number of eggs, the choice between one or other strategy may relate to environmental predictability, whereby laying more eggs per mass would be favoured in a habitat with low CR, while less eggs per mass would be expected to be laid in high CR habitats (Begon et al., 2006), although no consistent pattern was observed between adult survival differences in this study.

The high degree of correlation between F_1 and F_2 generations for both the number of egg masses produced and total egg number would suggest that these traits are under stronger genetic control than any of the other life history traits looked at thus far. This is as predicted by Stearns (1989a) who reported that life history traits more directly linked to fitness, such as those pertaining to reproduction and stage specific mortality, are more subject to stronger

selection pressures, being more likely to become canalised in rapid evolutionary time.

The observed differences in reproductive strategies are unlikely to be fully explained unless relationships between other life history traits (trade-offs) are considered (Stearns, 1989b). A more in depth analysis of trade-offs between traits will be carried out in Chapter 4, but it is important to highlight any apparent trends that appear when the individual traits are considered. For example the Epping population consistently produced both more eggs and more eggs per mass than other populations but did not produce more egg masses. The Epping population also suffered high juvenile mortality and the observed increase in reproductive output relative to other populations may serve as a trade-off against this high juvenile mortality.

Egg survivorship

Egg survivorship was shown to significantly vary at the population level between both the F_1 and F_2 generations. However, no consistent pattern of variation could be determined between generations. For example the Savernake population displayed the highest survivorship in the F_1 generation but lowest in the F_2. There were no marked differences between the range of survivorship values across generations, suggesting that egg survivorship did not suffer from effects of inbreeding as other life history traits.

Shell weight and calcium content

The formation of the shell in *L. stagnalis* requires the net uptake of environmental calcium, which in fresh water habitats can be as low as 1 to 3 mgL^{-1} (McMahon, 1983). *L. stagnalis* has historically been shown to be a calciphile species, typically not found in habitats with calcium concentrations below $20mgL^{-1}$ (Boycott, 1936) but has been shown to survive in sites with environmental calcium an order of magnitude below this value possibly due to an ability to supplement calcium through diet (Young, 1975). A radioactive tracer study by Van Der Borght and Van Puymbroek (1966) revealed that 80% of the calcium in the body was taken up from the media and the remaining 20% was derived from the lettuce the animals were fed on.

Another study by Greenaway (1971) also used radioactive tracers to examine the kinetics of calcium uptake and showed that *L. stagnalis* absorbed calcium directly from the surrounding medium, with absorption taking place via the epithelium which is selectively permeable to calcium ions. This author found that calcium uptake ran against a small electrochemical gradient at concentrations less than 0.5mM Ca^{2+}/L^{-1} (20mg Ca^{2+}/L^{-1}) and that at concentrations above this calcium uptake was either passive or actively favoured electrochemically. Greenaway (1971) also reported that at calcium concentrations ranging 1.0 to 1.5 mM Ca^{2+}/L^{-1} (40 to 60mg Ca^{2+}/L^{-1}) calcium uptake approached V_{max} saturation values. In this study both the low and high calcium concentrations were set at above the low end saturation value of 1.0 mM Ca^{2+}/L^{-1} and the absorption of calcium can be considered to be favoured electrochemically for both treatment groups. While this would suggest that the uptake of calcium by *L. stagnalis* is not particularly costly, McMahon (1983) states that as the resultant deposition of calcium in the shell runs against an electrochemical gradient, it is in shell deposition where the energetic costs of calcium uptake add up. McMahon (1983) goes on to suggest that relative differences in shell weights can be considered to reflect differences in energy expenditure between individuals.

In this study a linear relationship between shell weight and calcium content was established, whereby shell weight accounted for 96.5% of variation in calcium concentration. A study by Mackie and Flippance (1983) reported that when dried weights (shell and viscera) were compared in snails sampled across a range of environmental calcium concentrations that only 64.5% of calcium concentration could be predicted by dried total weight, but that environmental calcium concentration did not in any way correlate with the total calcium content of *L. stagnalis* as it did for some other mollusc species. This contrasts with Lewis and Magnusson (1999) who found that snails in calcium rich waters produced stronger shells with greater calcium content. The difference in correlations reported between this and the above study may be accountable to the fact that differences in site environmental calcium concentration may be detected in the visceral tissues of *L. stagnalis* and could explain the reduction of the R^2 value when combined visceral and shell weight

was compared. This hypothesis is also supported by Greenaway's (1971) findings that the tissues of *L. stagnalis* were found to be highly permeable to calcium, and it is expected that calcium content of visceral tissues may show correlation between site calcium concentration where shells do not, which could be tested by future experiment. Lodge *et al* (1987) suggest that the shell responses to calcium levels in the environment are complex and may be contingent on other factors such as predation risk which have not been quantified in this study.

The lack of any plastic response in shell calcium content to shell weight ratios across calcium regime would indicate that shell calcium content is genetically pre determined (canalised) and not subject to change in response to environmental variation. However, evidence for micro evolutionary differences in shell calcium content at the population level comes from the detection of significant inter population variation in shell calcium content, whereby the Epping population was shown to have significantly more shell calcium than the Brychfa and Fauldhouse populations (by ~ 0.2mg Ca/g shell weight $^{-1}$). The small range of difference in shell calcium content between populations would suggest that inter population differences in shell calcium concentration are particularly subtle and that, while some differences do exist between populations, dry shell weight is an appropriate (as well as cheaper and easier to measure) proxy for calcium concentration in *L. stagnalis*.

Length and age factored shell weights displayed a conserved response across calcium treatment over both generations with generally heavier shells being found in populations reared in high calcium treatments. Six populations (Bank, Brychfa, Glanahafren, Ireland, Savernake and Wreake) in the F_1 generation displayed significant intra specific differences in shell weight between calcium treatments, with consistently heavier shells being found in the high calcium treatment groups. The same trends were apparent in the F_2 generation but significant intra population differences were only detected for the Glanahafren and Wreake populations. This is in part due to the removal of the Bank, Fauldhouse and Savernake populations for further study, two of which displayed significant differences in the F_1 generation. The Epping,

Tiverton and Fauldhouse populations did not appear to display any plasticity in response to calcium treatment across generations.

Shell weight to length ratios were found to significantly correlate across generations with the Ireland and Epping populations displaying a consistent trend toward relatively lighter shells and the Glanaharen, and Wreake populations tending to have relatively heavier shells while the remaining populations displayed less consistent patterns. Such differences in relative shell weight could perhaps be due to different life history strategies driven by predation or in response to local selection in favour of other life history components other than shell thickness. The range of variation in shell weight to length ratios became greater in the high calcium group across both generations (Figure 2.69). This would suggest that genetic differences contributing to variation in shell weight across generations were conserved across generations, but that phenotypic plasticity displayed by some populations in response to environmental calcium concentration, resulted in a trend towards heavier shells being constructed in high calcium environments.

The results would support the view that shell weight to length ratio, is dictated by a direct relationship between environmental calcium concentrations in the majority but not all populations but an overall trend towards heavier shells being deposited in higher calcium media is apparent. McMahon (1983) suggests that a lack of response of some populations to environmental calcium may result from genetic drift or founder effects where alleles associated with calcium regulation become canalised over evolutionary time. This may be the case for the Fauldhouse population, which displays no response to calcium concentration with respect to shell growth and is known to have been introduced from another site, perhaps having been founded by a small number of individuals. The trends toward increased weight in the high calcium treatments across generations would also appear to closely mirror growth rates (parameter *c*) with higher growth corresponding to higher shell weight in the high calcium treatments, indicating a positive relationship between shell growth rate and shell weight. In this study it would appear that

the majority of snails reared in high calcium media grew faster and heavier than those reared in low calcium treatments.

Morphology

Freshwater gastropods are known to exhibit a high degree of morphological plasticity across geographic ranges (Mukaratirwa et al., 1998, Stothard et al., 1997), in response to exposure (Calow, 1981), and in response to local environmental cues such as predation by crayfish (DeWitt et al., 1999, Krist, 2002) or fish (Brönmark et al., 2011). A study by Arthur (1982) demonstrated that significant inter population differences in shell morphology of two distinct *L. stagnalis* field populations disappeared when animals were reared in the same laboratory conditions, highlighting the need to establish whether observed phenotypic differences are genetic or plastic in origin via laboratory based studies.

The PCA analysis of shell morphology in this study revealed that a large proportion of variation could be accounted for by PC1 across both generations (67% and 65% respectively). PC2 was found to account for less of the variation in the dataset (17% and 21% for F_1 and F_2 generations respectively) and would suggest that differences in morphology were more readily detected at the population level rather than in response to calcium treatment. Patterns of variation in component scores between populations and treatments varied between generations. Differences in morphology were more consistent between populations, but the effects of calcium availability were more evident in the F_2 generation. This suggests a greater response to calcium when other environmental influences have been excluded, but there are still significant differences in morphology between populations in the F_2 as well, indicating that the changes are not simply a result of plasticity. In the F_1 analysis the Fauldhouse population was found to have significantly higher PC1 scores than all other populations indicating that this population was morphologically distinct from the other populations by having comparatively wider shells with longer apertures.

As the Fauldhouse, Savernake and Bank populations were removed for further study the F_2 morphology analysis was only performed on data from the remaining six populations. When PCA analysis was performed the Epping and Wreake populations were shown to be morphologically distinct from some of the other populations, having narrower shells with shorter apertures relative to body length than the other populations. Significant intra population difference in PC1 score in response to calcium treatment was detected only for the Epping population which displayed a tendency towards narrower shells with shorter apertures when reared in low calcium media.

The PC1 loadings appear strongly consistent between the F_1 and F_2 generation (particularly when analysis of F_1 dataset was performed without the three populations removed for nanoparticle exposure) indicating that the observed morphological differences detected remain constant over time and are likely to be genetic in origin. The loadings of PC1 place strong emphasis on spire width:shell length and aperture length:shell length ratios indicating that population differences are best distinguished by variation in these morphological traits. Whether the Fauldhouse population would continue to remain morphologically distinct in the F_2 generation cannot be known. However given the strong degree of correlation of morphological traits (including shell width:shell length and body whorl length:shell length which loaded strongly on PC1) across F_1 and F_2 generations it is likely that this distinction would be conserved.

The F_2 PC2 analysis revealed a conserved response to calcium treatment indicating that snails reared in high calcium tended to have thicker spires relative to shell lengths than those in the low calcium treatments. It would appear that the effects of calcium treatment, like growth rates and relative shell weights, were more readily detectable in the F_2 generation. This suggests that the increased growth rates in the high calcium treatments resulted in heavier shells with thicker spires. That this trend is more readily detectable in the F_2 generation may be indicative of the breeding out of environmental effects present in the F_1 generation towards a more conserved response by the F_2 generation.

2.6. Conclusion

General conclusions

In general a highly complex pattern of life history trait variation was shown across the nine study populations. Different traits displayed different patterns of variation between populations and calcium treatments. Differences between populations were readily detected in most traits across both generations but consistent patterns proved more difficult to detect. As predicted by life history theory, life history traits with a strong connection to fitness (such as size at first reproduction, shell weight ratios, and reproductive outputs) tended to display high heritability (as indicated by stronger inter generational correlations) across generations (Roff and Mousseau, 1987). The response to calcium treatment varied for different traits and populations but appeared to be more conserved in traits directly related to growth and shell formation such as parameter *c*, shell weight ratios, shell morphology, age at first reproduction and reproductive output. This would suggest that different populations have possibly evolved independent life history strategies across different environments that have in part been influenced by environmental calcium availability, which varied strongly across sites sampled (see Figure 2.17). Evolved life history strategies may result from local environmental history representing different 'solutions' to distinct environmental challenges, but they also retain a significant degree of variability in most traits; a combination of genetic and plastic responses may allow the most effective adaptation of future environmental changes.

Many studies examining life history trait variation have focussed on freshwater snails and attempted to relate observed patterns of variation across and within genera to environments. An early study by Brown (1979a) revealed that four pulmonate snails, *Physa gyrinia*, *Physa integra*, *Lymnaea palustris*, and *L. stagnalis* employed distinctive life history strategies which were characterised by differences in growth rates, size and age at first reproduction, and fecundity. All four species displayed phenotypic plasticity in life history response to differences in temperature and density. The degree and magnitude of response was shown to vary across the species surveyed, and

the life history patterns observed were conserved across genera, indicating that observed differences had a genetic basis.

A later study also by Brown (1983) used discriminant analysis to examine whether differences in life history strategies were readily detectable at different levels of biological organisation from the family to the intra specific level. Differences in life history tactics proved to be more evident at higher taxonomic levels with differences between families being more readily detectable than those at the intra specific level. Nonetheless, Brown found significant differences between two different populations of *Lymnaea elodes*, sampled from temporary and permanent ponds (A and F respectively), which were characterised by differences in age and maturity and growth rate. A further study by this author (Brown, 1985) used reciprocal transfer experiments to establish that differences in these (and other) life history traits, while highly plastic in response to environmental differences, were heritable and therefore under genetic control. Brown found that snails from the permanent pond grew faster and a later study by Brown *et al* (1988) revealed that this population also suffered lower early mortality than snails from pond A. Most striking in Brown's account of *L.elodes* is the dramatic difference in reproductive cycles displayed between the two sites. Snails from pond A produced fewer eggs and displayed iteroparous reproduction, surviving to reproduce in a second year, while those from pond F displayed solely semelparous reproduction; producing an order of magnitude more eggs and dying after reproducing in the first year. Brown (1985) argues that in unpredictable habitats, phenotypic plasticity in life history traits itself could be subject to natural selection, whereby a range of possible phenotypes rather than one specific trait are selected for.

A field study by Lam and Calow (1989a) detected similar intra specific life history variation in *Lymnaea peregra* populations from three different sites. Life history differences in survivorship, growth and age and size at first reproduction were found between the different populations sampled. These authors found that differences in growth rates tended to be associated with site differences in water temperature while differences in age and size at first

reproduction were attributable to food availability at the particular sites. A further laboratory study by Lam and Calow (1989b) reared the field populations under constant laboratory conditions for two generations. This study revealed significant intra population differences in most life history traits across both the F_1 and F_2 generations. As with the results presented in this study patterns of variation varied within and between populations, and across generations. These authors attribute much of the observed variation to phenotypic plasticity however significant inter population differences in size at first reproduction were consistent with those observed in the field indicating that variation of this trait was under genetic control (Dillon, 2000). This mirrors the results presented in this study where size at first reproduction was shown to correlate across the F_1 and F_2 generations consistent with this trait being under genetic control. Reproductive output is shown to be a function of size at maturity in freshwater molluscs and the direct link of these traits to fitness may explain the low heritabilities presented in this and the above study (Dillon, 2000). Given the complexities in the patterns of life history variation shown in previous studies of a small number of populations (Brown, 1983, Brown et al., 1988, Lam and Calow, 1989a, Lam and Calow, 1989b) it is perhaps unsurprising that when attempting similar studies across a wider range of populations, clear-cut patterns are elusive.

Effects of environmental calcium

In general differences between life history traits were more readily detected at the population level than the intra population level in response to calcium treatment. This may be due to the fact that either calcium has little effect on life history traits (unlikely given the range of other studies that have demonstrated effects), that the threshold values for low and high calcium did not significantly challenge the sample populations to elicit a stress response, or that the thresholds for observed responses vary between populations due to underlying genetic differences. Conserved responses to calcium treatment were observed in some traits across all populations and tended to be found more in the F_2 generation suggesting that residual environmental effects in the F_1 generation masked a common response that was revealed by the F_2 generation. For example low calcium was shown to increase individual

mortality, decrease shell weight to length ratios, increase age at first reproduction, decrease growth rates and decrease clutch size (eggs per mass) in the F_2 generation. Given the large number of life history traits displaying a response (albeit a small one) to environmental calcium it seems likely that rather than life history traits not responding to calcium variation, that the range of environmental calcium concentrations used in this study was insufficient to challenge the animals and reveal the true extent of life history response to environmental calcium. It is likely that greater response to calcium treatment would be observed if the low calcium treatment was reduced to at least 20mgL^{-1} calcium or below where the physiological costs of calcium sequestration in *L. stagnalis* are greater (Greenaway, 1971). The sensitivity of mollusc life history traits to other variation in the environment complicates the interpretation of responses to calcium availability given the variation in other factors such as temperature regime, predator presence etc. that are also known to influence trait variation.

Intergenerational comparisons: Heritability and plasticity

The extent of trait variation between high and low calcium treatments is phenotypic plasticity, while the degree of similarity in traits across generations is the heritability, where high heritability implies a high degree of similarity in traits between offspring and parents (Price and Schluter, 1991).

It is possible for traits to be both plastic and to display low heritabilities. For example a trait could display plasticity across calcium treatments yet maintain the same overall value relative to other populations when inter generational comparisons were made. In this instance the degree of plasticity itself may be under selection in response to a number of hypothetical environments (Stearns, 2000). Observed plasticity can be the result of genotype x environmental interactions, of genetic differences between populations, or a combination of both these factors (Stearns, 1992).

Highly plastic traits with little heritability across generations

Traits pertaining to growth rates were shown to be highly plastic across populations and generations. For example parameter *c* values (representing

growth rates) were shown to be generally higher in the high calcium treatment across generations but the direction and magnitude of variation was shown to vary widely between populations and across generations. Parameter *c* values did not correlate significantly across generations, indicating that differences in growth rates are highly plastic in *L. stagnalis* and, while partly affected by environmental calcium, are subject to other environmental influences perhaps reflecting the high degree of environmental variation likely to be encountered by organisms in the field, and the need to maintain an ability to respond to such variation.

Plasticity in traits displaying heritability across generations

Size rather than age at first reproduction generally determines maturity in mollusc species and as this trait carries a direct link to fitness it would be expected to be under strong genetic control (Dillon, 2000). In this study considerable intra specific variation was displayed in this trait which was also shown to be phenotypically plastic in the F_1 generation with low calcium treatments reproducing at larger sizes. Plasticity in this trait was then shown to disappear by the F_2 generation but the intra specific differences were conserved across generations suggesting that differences at the population level were more genetic in origin and that initial plasticity in these traits was bred out under controlled conditions.

Similarly traits directly pertaining to reproduction, such as total egg mass number and total number of eggs, displayed a conserved response across generations. This may be an artefact of the fact that reproductive output in freshwater gastropods is shown to be a factor of adult size at maturity and the relationships between these life history traits will be further examined in Chapter 3. For the most part, like size at first reproduction, reproductive traits appeared to show distinct inter population variation that was conserved across generations with little plasticity in response to calcium levels. However, significantly more eggs per mass were laid in the high calcium groups by the F_2 generation suggesting some level of plasticity in reproductive traits in response to environmental calcium was detectable.

These findings support the view that high levels of plasticity are more likely to be observed in traits that respond directly to environmental variation such as growth rates, while plasticity would be expected to be reduced in those traits with a stronger link to fitness such as size at first reproduction and reproductive output, being more likely to show conserved responses across generations at the population level.

Individual life history strategies

The Epping, Savernake and Fauldhouse populations appeared to display some strong distinctions in life history strategy relative to other populations and are described below.

Epping

Hatchling groups in the Epping population displayed low growth followed by high growth in the linear phase for both generations. No response in growth with respect to calcium was noted in the F_1 generation but by the F_2 generation both growth rate and asymptotic shell size were found to be significantly higher in the high calcium treatment. The Epping population displayed high overall growth relative to other populations which also translated in this population attaining a generally larger final size. In addition high growth appeared to be correlated with high reproductive output both in terms of the total number of eggs produced and in terms of the number of eggs per mass. Epping was shown to display the highest reproductive output across generations for all the populations which supports the predictions of life history theory as described by Roff and Mousseau (1987) where lower heritabilites are found in traits directly associated with fitness. The high fecundities appeared to be offset against high juvenile mortality in both generations and it would seem that for this population there exists a trade-off between high juvenile mortality and fecundity.

Shell morphology and composition was shown to be distinct from other populations with the snails from the Epping site tending to be narrower with shorter apertures.

Shell calcium content was found to be significantly higher than other populations but shell weight to length ratios tended to be lower suggesting that shells from the Epping population contained more calcium but were relatively lighter than those from other populations. This would warrant further investigation to determine where the observed trends represent local microevolution in shell formation which may infer costs (trade-offs) in other life history traits (such as those observed in juvenile mortality). Increases in fitness from such an adaptation could be due simply from a reduction in the relative weight of the shell, freeing more energy to other life history components, or driven by ecological factors such as predation, where increased crush resistance due to higher shell calcium content results in improved fitness (Lewis and Magnuson, 1999). The former could be readily determined by experimentation to assess whether shells from the Epping population displayed greater crush resistance than those from other sites. The Epping population showed minimal response in shell weight to calcium treatment across both generations, indicating that little plasticity in calcium deposition across treatments exists for this population despite difference in growth rates in the F_2 generation. In summary the Epping population was found to have a life history strategy being characterised by a distinctive shell formation and large asymptotic growth corresponding to high adult fecundities with a possible trade-off between juvenile survivorship implied between these traits.

Fauldhouse

The Fauldhouse population displayed relatively high hatchling group growth in the F_1 generation, while a marked difference between calcium treatments was detected in the F_2 generation, where the low calcium treatment group was shown to grow significantly faster. Overall growth in the linear phase tended to be low relative to other populations with differences between calcium treatments only becoming apparent in the F_2 generation where snails grew faster in the high calcium treatments. Despite slow growth individuals grew to a large size taking relatively longer to reach first reproduction than those in other populations with highest ages at first reproduction occurring in the low calcium treatment groups.

The low growth rates displayed by the Fauldhouse population may be a local adaptation to low environmental calcium concentrations. Despite no differences in growth rates (parameter *c*) in the F_1 generation, the F_2 snails grew slightly slower in the low calcium treatments which translated to a large increase in age at first reproduction. The Fauldhouse population was shown to reproduce significantly later and at larger sizes in the low calcium treatments, with the F_2 generation displaying a marked reluctance to self supporting the observation that this introduced population may already be inbred. As with the Epping population large overall size at reproduction appears to be correlated with high reproductive output (both in terms of total eggs and eggs per mass) and a non significant trend to lower egg production in the low calcium treatment was observed. Like Epping, little response in shell weight to length ratios was noted across calcium treatment, again suggesting that no variability/plasticity was retained in this trait. The Fauldhouse population was found to display strong morphological distinctions from all other populations with shells from this population being characterised as being wider with bigger apertures. Like the shell calcium content differences displayed by the Epping populations, this may be indicative of micro evolution occurring in this population with respect to shell morphology. In summary the Fauldhouse population can be considered as having a life history strategy characterised by having distinctive shell morphology with a large size at first reproduction (and corresponding high fecundities) being attained via low growth rates. In this population the time to reach reproductive size was shown to be strongly affected by environmental calcium.

Savernake.

The Savernake population was shown to grow consistently faster in high calcium treatments across all life stages and generations. Growth differences resulted in the high calcium group growing faster, larger and heavier than the low calcium group. The low calcium group took significantly longer to reproduce in the F_1 generation with the same trend being apparent in the F_2 generation. The Savernake population was shown to have a smaller size at first reproduction relative to other populations which correlated in low

investment in reproduction, both in terms of a low total number of eggs and in low eggs per mass. Egg survivorship was found to be highly variable, being highest in the F_1 generation and lowest in the F_2 generation. These observations suggest that a trade-off between size at first reproduction and reproductive output has taken place with selection for early reproduction coming at the expense of a reduced number of offspring. The observed shift towards smaller sizes at first reproduction relative to other populations could be driven by size selective predation of adults and high survival of early life stages (Reznick et al., 1990). In summary the Savernake population displays a life history strategy characterised by low size at first reproduction with an implied trade-off between size at first reproduction and reproductive output that is mediated by environmental calcium concentration. Unlike the Epping and Fauldhouse populations the Savernake population displays strong plasticity in growth and size at first reproduction with respect to environmental calcium concentrations that is retained across generations. This would suggest that observed plasticity is in part genetic and that environmental instability in the form of fluctuations in site environmental calcium may have played a role in the evolved life history strategy for this population (Seigel and Ford, 2001).

Summary

The results presented in this chapter clearly indicate that *L. stagnalis* life history varies across the different populations sampled. It is apparent that even in controlled laboratory conditions that life history responses display a high degree of phenotypic plasticity but consistent patterns of variation detected in some traits between different populations across generations support the view that some component of life history trait variation is heritable. The Savernake, Fauldhouse and Epping populations appeared to display distinctive life history strategies that distinguished them from other study populations by differences in one or more life history traits.

Life history trait sensitivity to fluctuations in environmental calcium was noted across all populations with some conserved trends becoming apparent by the F_2 generation. However, the nature and magnitude of response was shown

to vary between and across populations, suggesting that like life history strategy responses to environmental calcium were both highly plastic and population specific. Analysis thus far has focussed on individual traits however in order to more fully understand the observed differences in population life history and response to environmental conditions a more full analysis of the relationship between traits, calcium treatment, and any trade-offs therein, will be performed in Chapter 3.

3. Evaluating differences in life history response to calcium availability at the population level: Trade-offs and population growth rates

3.1. Abstract

The effects of environmental calcium on life history traits and population growth rates over two generations in nine different populations of the great pond snail, *Lymnaea stagnalis*, were investigated. Multivariate analysis was used to determine the pattern and extent of life history trade-offs between populations and calcium treatments, while further analyses focussed on determining in which life history traits the majority of intra-specific variation could be found and the degree of sensitivity to environmental calcium levels. A stage-classified matrix model was constructed to derive population growth rates (λ) and elasticity analysis performed to examine the sensitivity of population growth rates to variation in different life history components and calcium levels.

A strong trade-off between age and size at first reproduction was detected across all generations and calcium treatments. Size at first reproduction was also shown to display strong positive correlation with reproductive output, wherein a further trade-off between eggs per mass and number of egg masses was detected. Trade-offs appeared to be best detected in traits with a stronger association with fitness and tentative evidence suggested that trade-offs were stronger under low calcium conditions.

The traits that best distinguished between population and calcium treatments varied between generations and differences were more pronounced in the F_2 generation indicating that underlying genetic difference between populations became more apparent as environmental influences were bred out.

A significant reduction in λ was detected across generations, most likely attributable to inbreeding. Elasticity analysis revealed that the reduction in λ was due to reduced early stage survivorship and lower adult fecundities. Calcium was found to have no significant effect on population growth although a trend towards lower population growth in the low calcium treatments was observed.

3.2. Introduction

Individual life history traits, and their response to calcium availability, show substantial variation between different populations of *L. stagnalis* (Chapter 2). A full understanding of the pattern of trait variation however requires consideration of the links between changes in different traits through trade-offs (Stearns, 1989b). Trade-offs are typically negative associations between two or more life history traits constrained via physiological or evolutionary processes. Without constraints imposed by trade-offs the world would be overrun by 'Darwinian demons' (Silvertown, 2005), hypothetical organisms with maximal fitness that reproduce immediately after birth, produce an infinite number of offspring and live for eternity. Trade-offs can be detected at different levels of biological organisation, from within individuals, to the population and species level (Stearns, 1992), and at different temporal stages within an organisms life (intra-individual trade-offs) extending beyond, to influence future generations (intergenerational trade-offs) (Stearns, 1989b).

Despite the importance of trade-offs in the understanding of life history theory, identifying and measuring them in the field can be confounded by a number of factors (Stearns, 1989b). Of principal importance is to determine whether any observed trade-off is genetic or phenotypic in origin and the implications that such difference may have in interpreting responses to selection over evolutionary time. The situation is further complicated due to the fact that phenotypic plasticity displayed in some life history traits may obscure trade-offs due to phenotypic covariance displayed across a number of environments (Pease and Bull, 1988). Despite the difficulties in measuring and interpreting trade-offs, several classic studies have examined common trade-offs in response to a number of different biological or abiotic mechanisms: between age and size at first reproduction in face of size specific predation pressures (Reznick, 1983, Stearns, 1983); between survivorship and reproductive investment (Cluttonbrock et al., 1983); and in response to latitudinal gradients (Olsson and Agren, 2002). Within lymnaeid snails, there are known trade-offs between investment in defence from predators and parasites and growth and reproductive traits (Rundle et al., 2004, Sandland and Minchella, 2004) with evidence of both phenotypic plasticity and genetic differentiation involved

in the observed responses, as well as trade-offs between growth and reproduction for individuals operating in different reproductive roles (Koene and Ter Maat, 2004).

Scaling up from the responses shown by individuals to population level effects in relation to overall population dynamics is also important to determine the potential longer-term significance in relation to population growth and selection. Matrix models have been widely used to translate from individual responses to population level effects in a variety of contexts: including: i.) analysing pod specific demography of killer whales, *Orcinus orca* (Brault and Caswell, 1993); ii.) evaluating conservation strategies for the endangered turtle *Clemmys guttata*; iii.) comparing plant life history responses (Enright et al., 1995); iv.) estimating fishing impacts on blue crab (*Callinectes sapidus*) populations (Miller, 2001), and v.) evaluating the effects of pollutants or toxicants on a number of different species (Sandrine et al., 2009, Billoir et al., 2007). In addition to determining the likely effects on population growth rate, such models also decompose the effects of individual components of an organisms life history (principally through examination of survival probabilities and time taken to pass through different life stages, plus fecundity) and determine how these may differentially influence population growth rate (Caswell, 2000).

Here, the data on variation in life history traits that were collected as part of the long-term experiments described in Chapter 2 were used to achieve three aims. Firstly to examine evidence for trade-offs between different traits and determine whether the pattern and extent of trade-offs varied between populations and calcium treatments; secondly to establish which life history traits contribute most strongly to the variation found. Thirdly to parameterise a stage-classified matrix model which was used to predict population growth rates across the different treatments and populations and examine the sensitivity of growth rates to variation in different life history components.

3.3. Materials and methods

3.3.1. Source of data

All analyses were performed on the data derived from F_1 and F_2 generations described in Chapter 2.

3.3.2. Multivariate analyses of trade-offs between traits

Principal component analysis

Relationships between life history traits and any trade-offs therein were analysed using Principal Components Analysis (PCA) using Minitab V.15 for Windows. PCA relies on orthogonal transformation of a set of variables to derive a set of linear uncorrelated variables (principal components) where patterns of variation in the derived components are based on correlations between the original variables (Tabachnick and Fidell, 2001). In the context of this study PCA is of use in highlighting trade-offs between traits as it allows the major patterns of variation in the data to be examined with respect to different variables (population and calcium treatment).

The following traits were used as inputs for the PCA: Total eggs in 21 days, Total eggs masses in 21 days, Eggs per mass, Egg survival and Size and Age at 1st reproduction. Initial analysis considered the entire dataset for each generation and differences between populations and calcium treatments in PC1 and PC2 were determined using ANOVA.

In order to assess whether any differences in trade-off structure were apparent under different levels of environmental stress PCA was also performed in the high and low calcium treatments for both generations separately.

Discriminant function analysis

To assess the extent to which populations could be separated on the basis of significant differences in trait values, and whether the separation varied between calcium treatments, Stepwise Linear Discriminant Function Analysis (DFA) ($F<P=0.05$ for variable inclusion in all cases) was performed using

SPSS V.15 for Windows. DFA is used to determine which variables best separate or distinguish between groups. Variables are given loadings (positive or negative) which correspond to the strength of separation in each function. The majority of variability in the dataset is typically accounted for by the first two functions (Tabachnick and Fidell, 2001). In the context of this study DFA allows the traits which best separate at the population or calcium treatment level to be determined.

Analyses were performed on all populations from the F_1 and F_2 high and low calcium datasets combined and also each generation separated by calcium treatment.

Due to the fact that the Fauldhouse, Bank Well and Savernake populations had been retained for further study and were not subjected to culling after 21 days of reproduction, it was not possible to gather shell weight to length data as it had been for the other F_2 populations. As shell weight to length ratio had been proven to play a strong role in discriminating groups in the F_1 dataset, the same discriminant analysis was also performed on the F_2 dataset with the populations used in CB NP analyses (see Chapter 4) omitted to allow direct comparison between generations.

3.3.3. Matrix model development

A stage-classified matrix model was constructed to assess how predicted population growth varied between populations and calcium treatments. Four life cycle stages were defined for model development: eggs, hatchlings, juveniles and adults. The length of the egg stage was defined by the mean period of time after the eggs were laid until they hatched. Hatchlings and juveniles were analysed separately in the experiments and in the model on the basis that previous observations had shown that mortality was relatively high in the period immediately after hatching, but reduced as individuals grew larger. The hatchling stage was considered to last from hatching until when individuals reached the mean initial size of individuals used in the juvenile exposure group. The juvenile stage was considered to last from the end of

the hatchling stage to the time when individuals reached the mean initial size of individuals used in the adult exposure group.

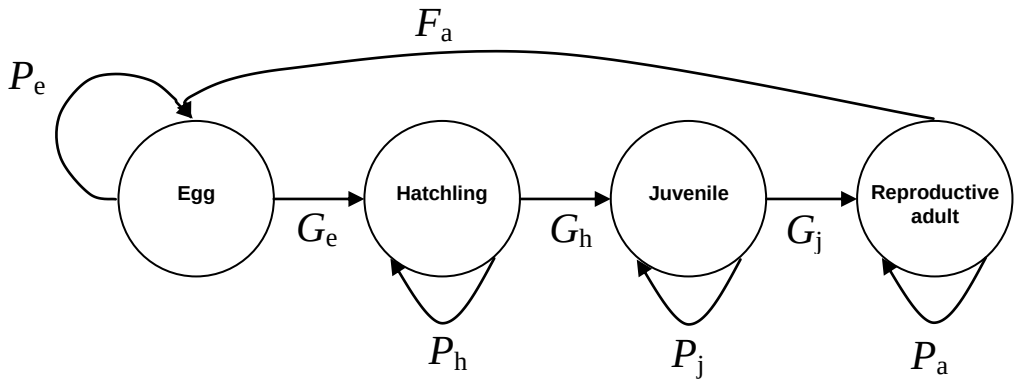


Figure 3.1. Stage based life history diagram for *L. stagnalis*.

The four life cycle stages are represented in the life history diagram displayed in Figure 3.1. This diagram was then used to create the projection matrix, *A* (3.1), where the P_i, G_i and F_i values correspond to those in Figure 3.1. The P_i values are transition probabilities and correspond to the probability of surviving a given growth stage while the G_i values represent the probability of surviving and growing to the next stage. F represents the fecundity (individual reproductive output per time step). The model had a time step of one week and was constructed and analysed according to the methods and equations described for stage classified matrix construction by Caswell (2000).

$$A = \begin{pmatrix} P_e & 0 & 0 & F_a \\ G_e & P_h & 0 & 0 \\ 0 & G_h & P_j & 0 \\ 0 & 0 & G_j & P_a \end{pmatrix} \quad (3.1.)$$

The transition probabilities were calculated from estimates of the per timestep probability of individuals in a given stage surviving, σ_i, and the per timestep probability of an individual entering the next life stage γ_i.

Values of σ_i were calculated according to the following equation (Equation 3.2):

$$\sigma_i = e^{\frac{ln(s_i)}{t}} \tag{3.2.}$$

where s_i represents the mean proportional survival at each stage, and t is the time over which the stage lasted (in weeks).

Values of γ_i were calculated according to the method given by Caswell (2000) for variable stage durations (equation 3.3):

$$\gamma_i \approx \left(\frac{1}{\bar{T}_i}\right) exp\left(-a\left(\frac{\bar{T}_i}{2} - \frac{V(\bar{T}_i)}{2\bar{T}_i}\right)\right) \tag{3.3.}$$

where $\bar{T}_i$ represents the mean time spent in a given stage and V represents the variance associated with that mean.

These estimates were combined to provide stage-based survival and transition probabilities for the matrix model according to Equations 3.4 and 3.5:

$$G_i = \sigma_i \gamma_i \tag{3.4.}$$

$$P_i = (1 - \gamma_i) \tag{3.5.}$$

The fecundity term, F_a, was defined as the mean number of eggs produced per individual per week multiplied by adult survivorship.

Matrix *A* was then subjected to eigenanalysis to derive values of the population growth rate, λ. Confidence intervals for λ were derived via bootstrapping (Caswell, 2000). In order to assess the relative importance of different matrix parameters to overall λ, *A* was then subject to elasticity analysis to determine the relative change in λ resulting from equal proportional changes in each of the matrix parameters. This allows the relative sensitivity of overall growth rate to each of the different matrix parameters to be examined. Elasticity was defined by Equation 3.6:

$$e_{ij} = \frac{a_{ij}}{\lambda} \frac{\delta\lambda}{\delta a_{ij}} \quad (3.6.)$$

where a_{ij} is an element of matrix A.

Eigenanalysis of all life history matrices and subsequent bootstrapping via Monte Carlo analysis was performed using the PopTools V.3.2. add-in for Excel.

3.4. Results

3.4.1. Multivariate analysis: Principal component analyses

3.4.1.1. F_1 generation life history trait analysis via PCA

The results of PCA carried out on the F_1 dataset are summarised in Table 3.1 and a corresponding plot for the first two components is displayed in Figure 3.2.

Table 3.1. Results of PCA analysis for life history traits, F_1 generation.

Axis	PC1	PC2	PC3
Eigenvalue	2.232	1.369	1.004
Proportion	0.372	0.228	0.167
Cumulative	0.372	0.600	0.768
Variable	**PC1**	**PC2**	**PC3**
Total eggs in 21 days	0.643	0.142	-0.025
Egg masses in 21 days	0.500	-0.014	0.159
Mean eggs per mass	0.471	0.265	-0.277
Age 1st reproduction	0.042	-0.761	0.043
Mean survival to hatching	-0.071	-0.115	-0.946
Length at 1st reproduction	0.329	-0.562	-0.007

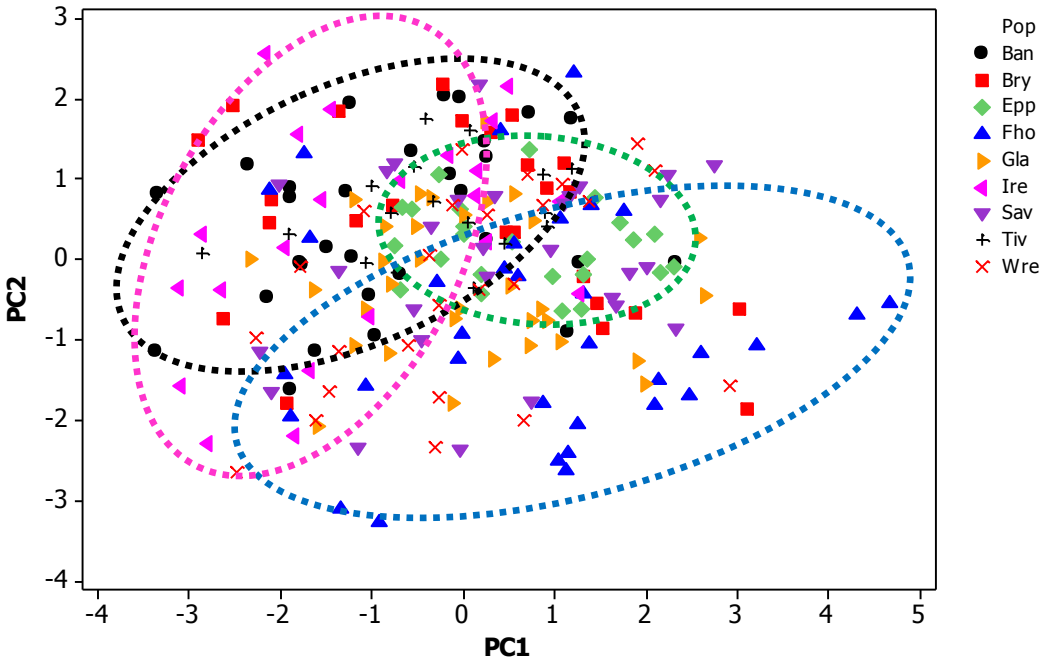


Figure 3.2. PCA analysis of life history traits F_1 generation high and low calcium combined. Principal component 1 vs. Principal component 2. Single data points are derived from individual snails, while dashed circles denote populations found to display significant differences from other populations.

ANOVA analysis was performed on scores of components 1 and 2 and the results are displayed in Table 3.2. Bank Well (black dot, circled in Figure 3.2) population was found to significantly differ from the Epping (green diamond, circled), Fauldhouse (blue triangle, circled) and Savernake populations (Tukey test, $P<0.05$). The Ireland (pink triangle, circled) population was also found to differ significantly from the Epping, Fauldhouse and Savernake populations in PC1 (Tukey test, $P<0.05$).

Table 3.2. Summary ANOVA table for individual components of PCA analysis of life-history traits, F_1 generation.

PC1 Score	D.F	F Statistic	P-value	Significant
Population	8, 220	4.96	<0.001	Y
Calcium	1, 220	2.77	0.097	N
Population * Calcium	8, 220	0.81	0.590	N
PC2 Score	**D.F**	**F Statistic**	**P-value**	**Significant**
Population	8, 220	5.97	<0.001	Y
Calcium	1, 220	22.51	<0.001	Y
Population * Calcium	8, 220	7.81	<0.001	Y

F_1 low calcium treatments PCA

The results of PCA carried out on the F_1 low calcium dataset are summarised in Table 3.3. The corresponding loading plot for the first two components is displayed in Figure 3.3.

Table 3.3. Results of PCA analysis for life history traits, F_1 generation, low calcium treatment group.

Axis	PC1	PC2	PC3
Eigenvalue	2.381	1.361	0.669
Proportion	0.397	0.227	0.161
Cumulative	0.397	0.624	0.784
Variable	**PC1**	**PC2**	**PC3**
Total eggs in 21 days	0.623	0.096	0.122
Egg masses in 21 days	0.511	-0.122	0.212
Mean eggs per mass	0.483	0.290	0.008
Age 1st reproduction	-0.104	-0.661	0.373
Mean survival to hatching	-0.165	0.285	0.895
Length at 1st reproduction	0.265	-0.611	-0.006

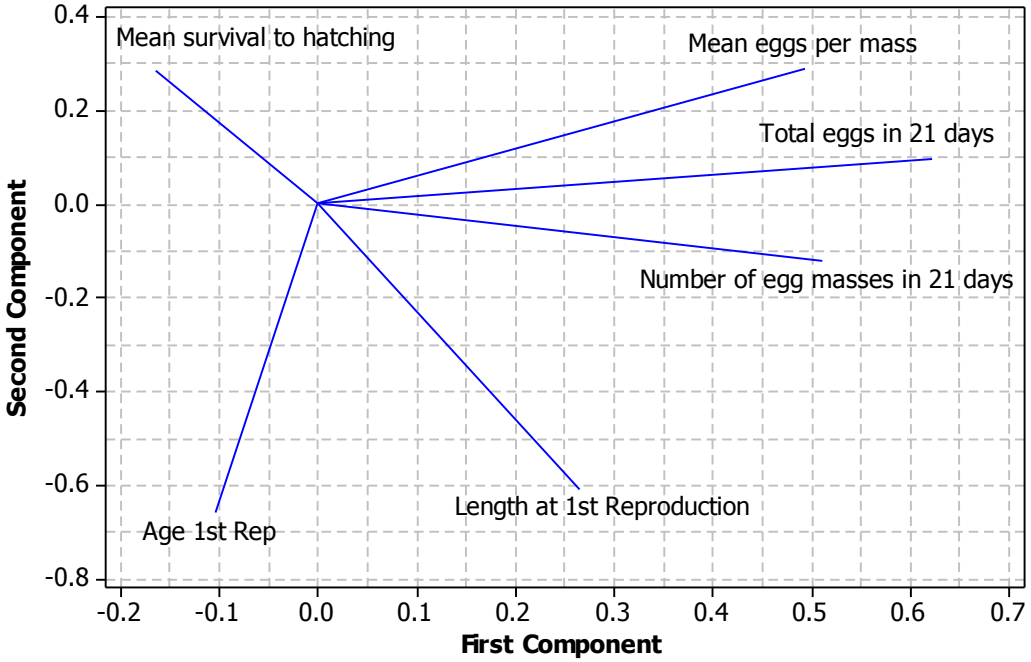


Figure 3.3. PCA loading plot for F_1 life history traits for all populations in the low calcium treatment group.

F_1 high calcium treatments PCA

The results of principal PCA carried out on the F_1 high calcium dataset are summarised in Table 3.4. The corresponding loading plot for the first two components is displayed in Figure 3.4.

Table 3.4. Results of PCA analysis for life history traits, F_1 generation, high calcium treatments.

Axis	PC1	PC2	PC3
Eigenvalue	2.223	1.425	1.117
Proportion	0.371	0.238	0.186
Cumulative	0.371	0.608	0.794
Variable	**PC1**	**PC2**	**PC3**
Total eggs in 21 days	0.611	0.309	-0.051
Egg masses in 21 days	0.448	0.248	-0.568
Mean eggs per mass	0.418	0.204	0.633
Age 1st reproduction	0.197	-0.599	-0.398
Mean survival to hatching	0.144	-0.493	0.317
Length at 1st reproduction	0.439	-0.447	-0.122

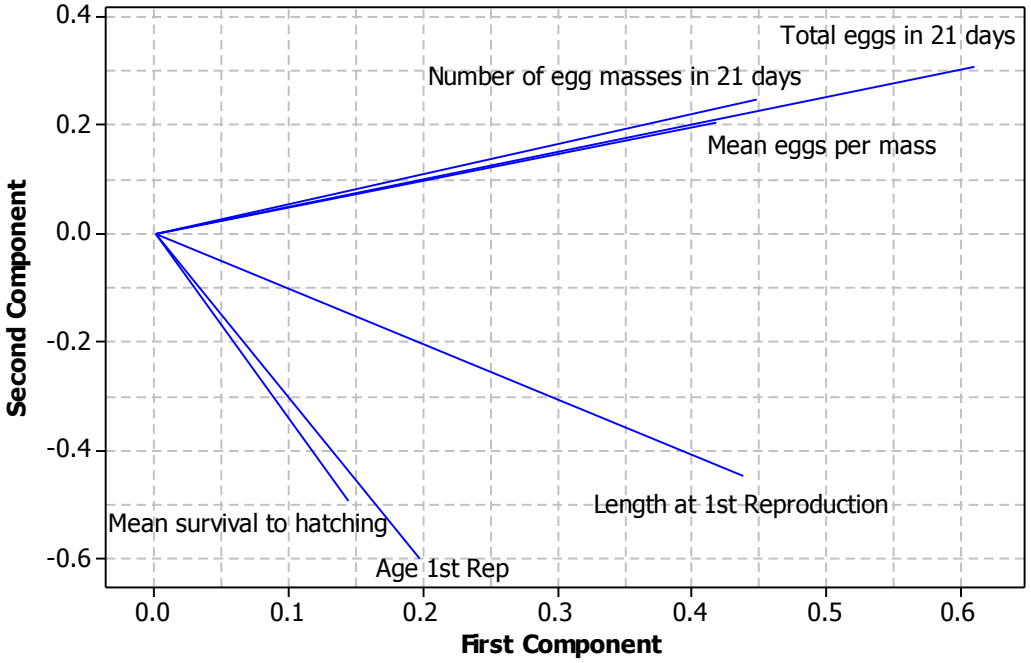


Figure 3.4. PCA loading plot for F_1 life history traits for all populations in the high calcium treatment group.

3.4.1.2. F_2 generation life history trait analysis via PCA

The results of PCA carried out on the F_2 dataset are summarised in Table 3.5 and a corresponding plot for the first two components is displayed in Figure 3.5 while figure 3.6 displays the mean PC1 values for each population to illustrate where inter-population differences lie. ANOVA analysis was performed on scores of components 1 and 2 and the results are displayed in Table 3.6.

Table 3.5. Results of PCA analysis for life history traits, F_1 generation.

Axis	**PC1**	**PC2**	**PC3**
Eigenvalue	2.252	1.223	1.013
Proportion	0.375	0.204	0.169
Cumulative	0.375	0.579	0.748
Variable	**PC1**	**PC2**	**PC3**
Total eggs in 21 days	-0.619	-0.099	0.126
Egg masses in 21 days	-0.291	-0.725	0.358
Mean eggs per mass	-0.488	0.515	-0.149
Age 1st reproduction	0.199	-0.243	-0.638
Mean survival to hatching	-0.164	-0.368	-0.582
Length at 1st reproduction	-0.476	0.070	-0.296

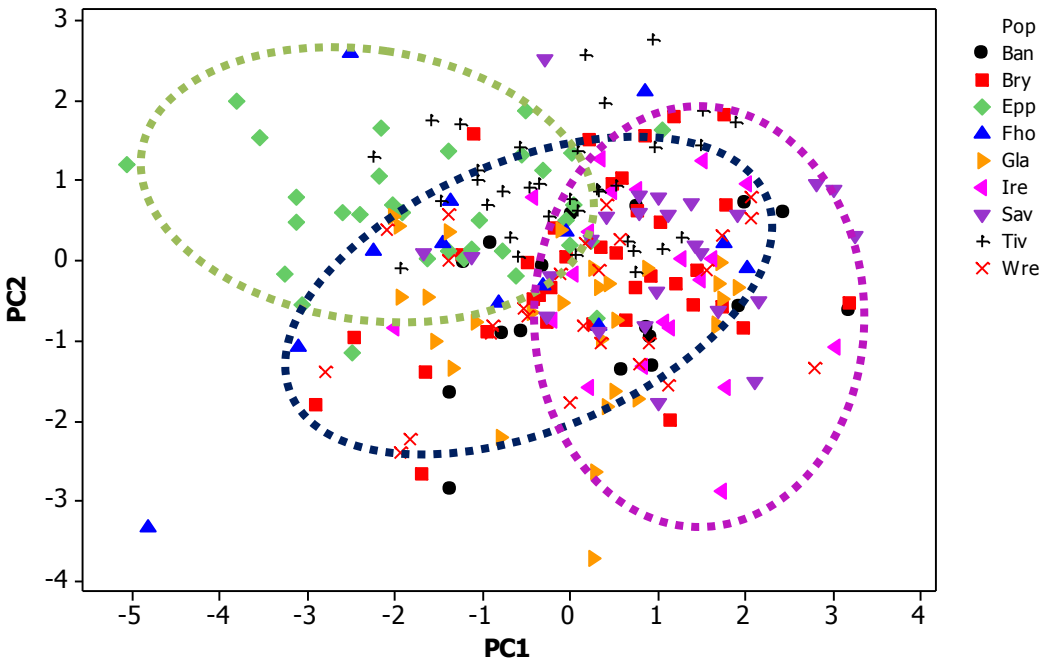


Figure 3.5. PCA analysis of life history traits F_2 generation high and low calcium combined. Principal component 1 vs. Principal component 2. Single data points are derived from individual snails, while dashed circles denote populations found to display significant differences from other populations.

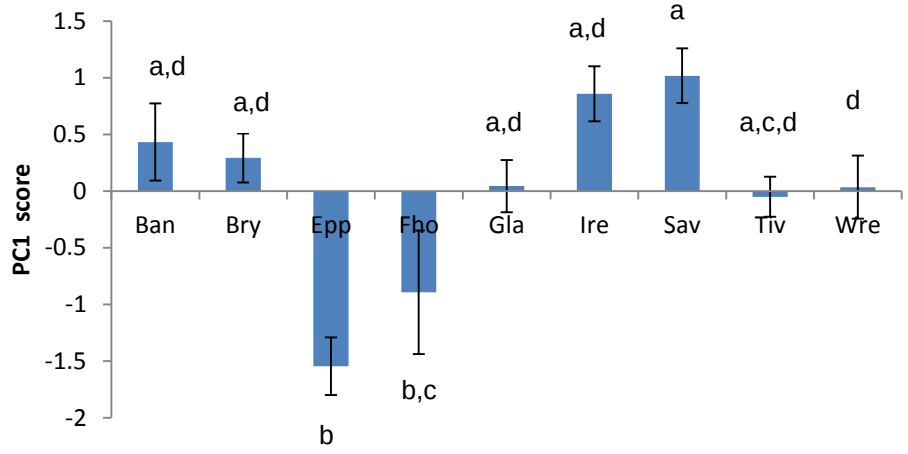


Figure 3.6. Mean PC1 scores for F_2 generation high and low calcium treatments combined (same letters denote populations with no significant differences between means). Error bars denote standard error of mean.

From post hoc testing of the PC1 scores (see Figures 3.5 and 3.6) the Epping (green diamonds, circled in Figure 3.5) and Fauldhouse (blue triangles,

circled) populations were found to have significantly lower PC1 scores than the other populations. The Savernake population (purple triangles, circled) was found to differ from the Wreake population by having higher PC1 scores (Tukey test, $p<0.05$).

Table 3.6. Summary ANOVA table for individual components of PCA analysis of life-history traits, F_2 generation.

PC1 Score	D.F	F Statistic	P-value	Significant
Population	8, 214	11.37	<0.001	Y
Calcium	1, 214	0.15	0.695	N
Population * Calcium	8, 214	2.77	0.006	Y
PC2 Score	**D.F**	**F Statistic**	**P-value**	**Significant**
Population	8, 214	11.43	<0.001	Y
Calcium	1, 214	12.70	<0.001	Y
Population * Calcium	8, 214	0.63	0.750	N

F_2 low calcium treatments PCA

The results of principal PCA carried out on the F_2 low calcium dataset are summarised in Table 3.7. The corresponding loading plot for the first two components is displayed in Figure 3.7.

Table 3.7. Results of PCA analysis for life history traits, F_2 generation, low calcium treatment group.

Axis	PC1	PC2	PC3
Eigenvalue	2.473	1.361	0.903
Proportion	0.412	0.227	0.151
Cumulative	0.412	0.639	0.790
Variable	**PC1**	**PC2**	**PC3**
Total eggs in 21 days	-0.565	-0.258	0.140
Egg masses in 21 days	-0.222	-0.722	0.354
Mean eggs per mass	-0.502	0.352	-0.087
Age 1st reproduction	0.374	-0.326	0.180
Mean survival to hatching	-0.147	-0.403	-0.885
Length at 1st reproduction	-0.467	0.143	0.178

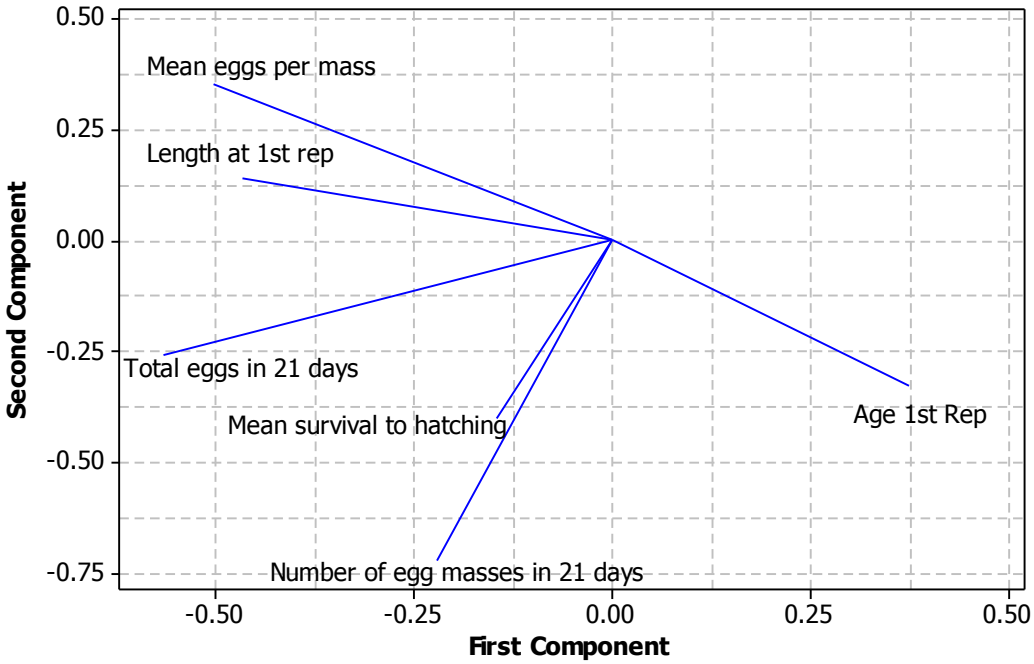


Figure 3.7. PCA loading plot for F_2 life history traits for all populations in the low calcium treatment group.

F_2 high calcium treatments PCA

The results of principal PCA carried out on the F_2 high calcium dataset are summarised in Table 3.8. The corresponding loading plot for the first two components is displayed in Figure 3.8.

Table 3.8. Results of PCA analysis for life history traits, F_2 generation, high calcium treatments.

Axis	PC1	PC2	PC3
Eigenvalue	2.224	1.180	1.167
Proportion	0.371	0.197	0.195
Cumulative	0.371	0.567	0.762
Variable	**PC1**	**PC2**	**PC3**
Total eggs in 21 days	0.630	-0.026	0.180
Egg masses in 21 days	0.345	-0.716	0.034
Mean eggs per mass	0.467	0.570	0.201
Age 1st reproduction	-0.049	0.316	-0.643
Mean survival to hatching	0.155	-0.227	-0.647
Length at 1st reproduction	0.490	0.097	-0.306

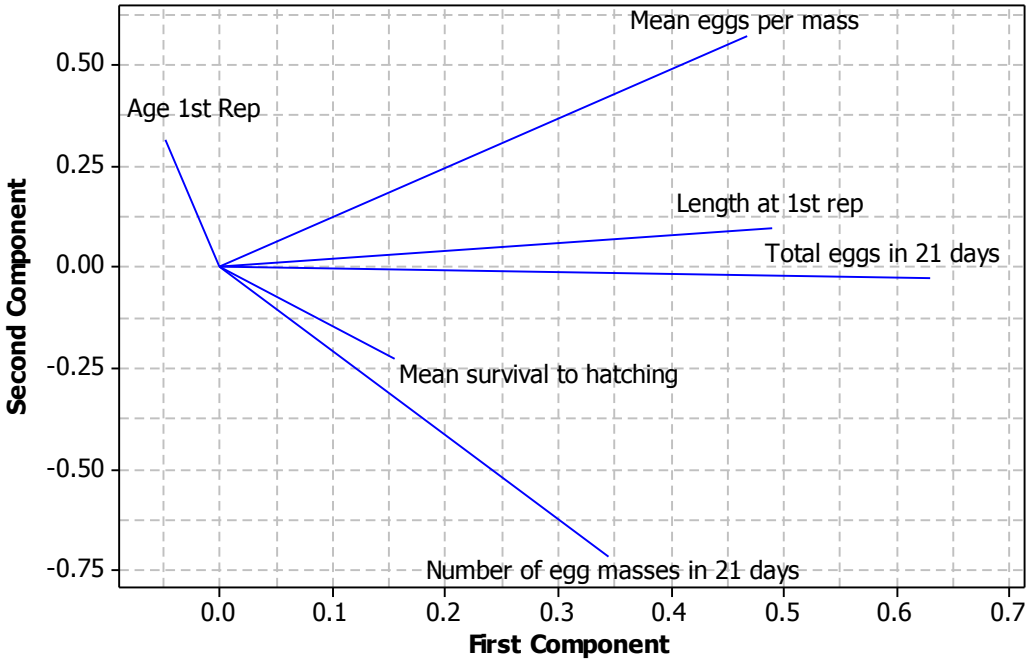


Figure 3.8. PCA loading plot for F_2 life history traits for all populations in the high calcium treatment group.

3.4.2. Multivariate analysis: Discriminant function analyses

3.4.2.1. F_1 life history trait analysis via discriminant function analyses

Discriminant function analysis allowed 41.8% of all populations to be classified correctly (see Figure 3.9). The results of discriminant analysis are displayed in Table 3.9 where it can be seen that 81.9% of the variation in the dataset could be explained by the first 3 functions. As over 70% of the variance was restricted to the first two functions, interpretation was restricted to these for simplicity.

Table 3.9. Results of discriminant analysis for life history traits, F_1 generation.

Function	Eigen value	Percent of variance	Cumulative Variance	Canonical correlation	Wilk's Lambda	Significance
1	1.682	47.9	47.9	0.792	0.083	<0.001, Y
2	0.786	22.4	70.3	0.663	0.223	<0.001, Y
3	0.408	11.6	81.9	0.538	0.398	<0.001, Y

Standardised discriminant coefficients for the first two functions are displayed in Table 3.10 while the structure matrix is displayed in Table 3.11. From Table 3.10 it can be seen that length at first reproduction made the largest positive contribution to the first function, followed by a positive contribution from age at

1st reproduction, and negative contribution from weight to length. Number of egg masses in 21 days, total eggs in 21 days and parameter *c* were not selected during stepwise selection. The second function displayed the largest absolute positive correlation between length at 1st reproduction, weight to length and the number of egg masses in 21 days. This indicates differences between these traits may be best observed in function 2. Parameter *b* was also shown to negatively correlate with the second function.

Table 3.10. Standardised canonical discriminant function coefficients for F_1 generation, high and low calcium.

Life history trait	Function	
	1	2
Parameter *a*	-0.260	0.049
Parameter *b*	0.133	-0.158
Mean eggs per mass	0.097	-0.401
Mean survival to hatching	0.026	-0.165
Age 1st reproduction	0.328	-0.404
Length at 1st reproduction	1.138	0.640
Weight to length	-1.038	0.572

Table 3.11. Structure matrix displaying pooled within-group correlations between discriminating variables for first two functions with variables ordered by absolute size of correlation within function for F_1 life history data. Asterisk (*) denotes largest absolute correlation between any variable and discriminant function while *a* indicates variables rejected from analysis.

Life history trait	Function	
	1	2
Length at 1st reproduction	0.521	0.793*
Weight to Length	-0.262	0.778*
Number of egg masses in 21 days[a]	-0.029	0.216*
Age 1st reproduction	0.355	0.088
Mean eggs per mass	0.090	-0.032
Total eggs in 21 days[a]	0.072	0.093
Parameter *b*	0.101	-0.258
Parameter *c*[a]	-0.141	0.140
Mean survival to hatching	-0.071	-0.085
Parameter *a*	0.007	0.323

The results of the discriminant analyses are displayed in Figure 3.9.

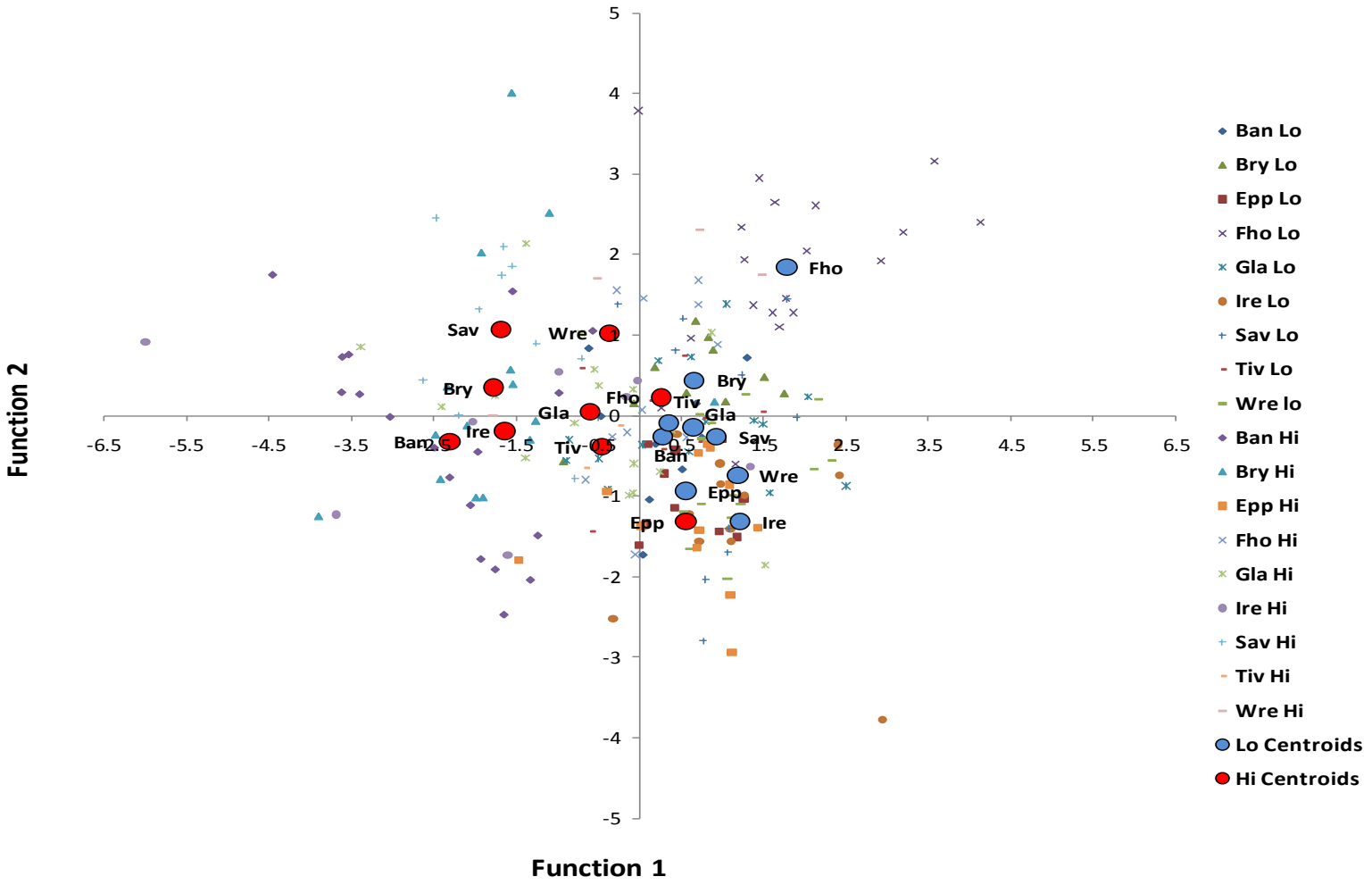


Figure 3.9. Results of discriminant analyses of entire F_1 life history dataset. Coefficients for Function 1 and 2 are given in Table 3.10.

From Figure 3.9 it is apparent that the low calcium populations are found to have more positive values on Function 1, while the high calcium group tend towards negative values. As length and age at first reproduction were positively correlated with Function 1 (Table 3.11) this would suggest that the low calcium populations are longer and older at first reproduction than the high calcium populations. As weight to length was negatively correlated with Function 1 this would indicate that the low calcium populations had lower weight to length ratios and therefore were characterised by being lighter per unit length than the high calcium populations. The low calcium Fauldhouse population displays clear separation from the other populations and with respect to Function 1 can be considered to be the population most defined by a combination of high age and length at first reproduction and lowest weight to length ratio. Highest group classification was recorded in the high calcium Tiverton and Savernake groups group (with 100% and 70% of individuals correctly classified respectively) and the low calcium Fauldhouse group with 73.7% of individuals classified correctly.

F_1 low calcium populations, discriminant function analyses

Discriminant analysis allowed for 47.9% of all populations to be classified correctly (see Figure 3.10). The results of discriminant analysis are displayed in Table 3.12 where it can be seen that nearly 90% of the variation in the dataset could be explained by the first 3 functions. As before analysis focussed on the first 2 functions which accounted for 80% of the variance in the dataset.

Table 3.12. Results of discriminant analysis for life history traits, F_1 generation, low calcium.

Function	Eigen value	Percent of variance	Cumulative Variance	Canonical correlation	Wilk's Lambda	Significance
1	1.500	63.0	63.0	0.775	0.184	<0.001, Y
2	0.407	17.1	80.1	0.538	0.459	<0.001, Y
3	0.220	9.2	89.3	0.425	0.645	0.001, Y

Standardised discriminant coefficients for the first two functions are displayed in Table 3.13 while the structure matrix is displayed in Table 3.14. From Table 3.14 it can be seen that length at first reproduction made the largest positive contribution to the first function, followed by a positive contribution from weight

to length and thirdly age at 1st reproduction. Both length at first reproduction and weight to length variables were found to make the largest absolute contribution to the first function, indicating that differences between these traits will be best observed in Function 1.

The second function displayed the largest absolute positive correlation between growth parameter *b* and strong positive correlation between age at 1st reproduction. This indicates population differences between initial size (growth parameter *b*) may be best observed in function 2.

Table 3.13. Standardised canonical discriminant function coefficients for F_1 low calcium treatments.

Life history trait	Function	
	1	2
b2	0.130	0.826
Mean eggs per mass	-0.244	0.047
Age 1st reproduction	-0.272	0.572
Length at 1st reproduction	0.774	0.144
Weight to length	0.484	-0.212

Table 3.14. Structure matrix displaying pooled within-group correlations between discriminating variables for first two functions with variables ordered by absolute size of correlation within function for F_1 low calcium life history data. Asterisk (*) denotes largest absolute correlation between any variable and discriminant function while *a* indicates variables rejected from analysis.

Life history trait	Function	
	1	2
Length at 1st reproduction	0.888*	0.003
Weight to Length	0.770*	0.013
Parameter *a*[a]	0.434*	0.332
Parameter *b*	-0.102	0.842*
Parameter *c*[a]	0.071	-0.553*
Mean survival to hatching[a]	-0.017	-0.028
Mean eggs per mass	-0.044	-0.130
Total eggs in 21 days[a]	-0.034	-0.126
Number of egg masses in 21 days[a]	0.077	-0.166
Age 1st reproduction	0.211	0.548

The results of the discriminant analyses for the F_1 low calcium populations are displayed in Figure 3.10.

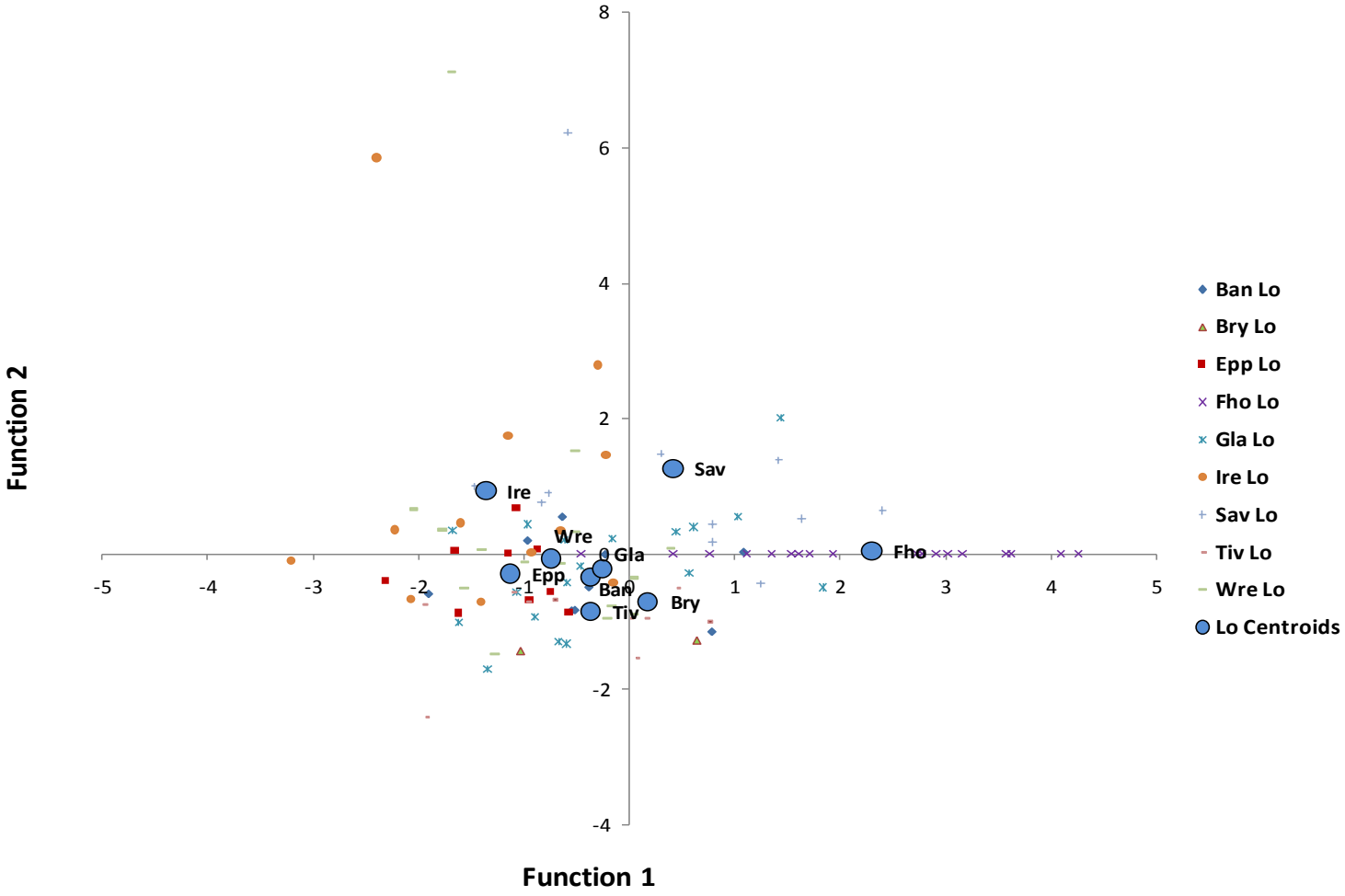


Figure 3.10. Results of discriminant analyses of low calcium populations in F_1 life history dataset. Coefficients for Function 1 and 2 are given in Table 3.13.

From Figure 3.10. as in figure 3.9. the low calcium Fauldhouse population is again shown to stand out distinctly from the other populations, being characterised by higher positive values in Function 1.

As length and age at 1st reproduction and weight to length were positively correlated with Function 1 (Table 3.14) this would suggest that of the low calcium populations, individuals from the Fauldhouse population tend to take longer to reach reproduction which they do so at a larger size and with a higher weight to length ratio than the other populations. It would appear that when the low calcium populations are analysed alone that the weight to length ratio switches from being weakly negatively to strongly positively correlated with length and age at first reproduction (see Tables, 3.11 and 3.14) indicating that differences in shell weight and length may affect other life history traits more strongly in the low calcium populations than in their high calcium counterparts. The Savernake and (to a lesser extent) Ireland populations appear to display separation from most of the other groups in Function 2. As parameter *b* and age at 1st reproduction were correlated most strongly in function 2 these populations can be considered to be defined as differing from the others in having larger initial size/rate of growth and higher age at first reproduction. Conversely those populations nearer or below the x-axis (Epping, Fauldhouse, Wreake, Bank , Glanahafren, Tiv and Brychfa) would be expected to have smaller initial sizes/growth rates and reproduce earlier.

Highest group classification was recorded in the Fauldhouse, Epping and Brychfa populations, with 80%, 72.7% and 60% of individuals correctly classified respectively.

F_1 high calcium populations, discriminant function analyses

Discriminant analysis allowed for 47.7% of all populations to be classified correctly (see Figure 3.11). The results of discriminant analysis are displayed in Table 3.15 where it can be seen that 82.4% of the variation in the dataset could be explained by the first 3 functions. As before analysis focussed on the first 2 functions which accounted for 65.2% of the variance in the dataset.

Table 3.15. Results of discriminant analysis for life history traits, F_1 generation, low calcium.

Function	Eigen value	Percent of variance	Cumulative Variance	Canonical correlation	Wilk's Lambda	Significance
1	0.936	45.0	45.0	0.695	0.184	<0.001, Y
2	0.421	20.2	65.2	0.544	0.459	<0.001, Y
3	0.356	17.1	82.4	0.513	0.645	<0.001, Y

Standardised discriminant coefficients for the first two functions are displayed in Table 3.16 while the structure matrix is displayed in Table 3.17. From Table 3.17 it can be seen that age at first reproduction and length at first reproduction make the two largest positive contributions to the first function followed by a weaker positive contribution from mean eggs per mass. Weight to length was found to make a weaker negative contribution, suggesting that this variable was strongly positively correlated with age and length at first reproduction only in the low calcium groups (see Table 3.14).

The second function displayed the largest absolute positive correlation between length at first reproduction and weight to length.

Table 3.16. Standardised canonical discriminant function coefficients for F_1 high calcium treatments.

Life history trait	Function	
	1	**2**
Parameter *c*	-0.223	0.341
Mean eggs per mass	0.542	-0.274
Age 1st reproduction	0.731	-0.210
Length at 1st reproduction	0.559	0.758
Weight to length	-0.917	0.425

Table 3.17. Structure matrix displaying pooled within-group correlations between discriminating variables for first two functions with variables ordered by absolute size of correlation within function for F_1 high calcium life history data. Asterisk (*) denotes largest absolute correlation between any variable and discriminant function while a indicates variables rejected from analysis.

Life history trait	Function	
	1	**2**
Length at 1st reproduction	0.404	0.874*
Weight to Length	-0.258	0.803*
Total eggs in 21 days[a]	0.113	0.363*
Number of egg masses in 21 days[a]	-0.083	0.325*
Mean survival to hatching[a]	0.012	0.156*
Age 1st reproduction	0.506	0.336
Mean eggs per mass	0.298	0.126
Parameter *c*	-0.027	0.296
Parameter *a*[a]	0.072	0.388
Parameter *b*[a]	-0.023	-0.286

The results of the discriminant analyses for the F_1 low calcium populations are displayed in Figure 3.11.

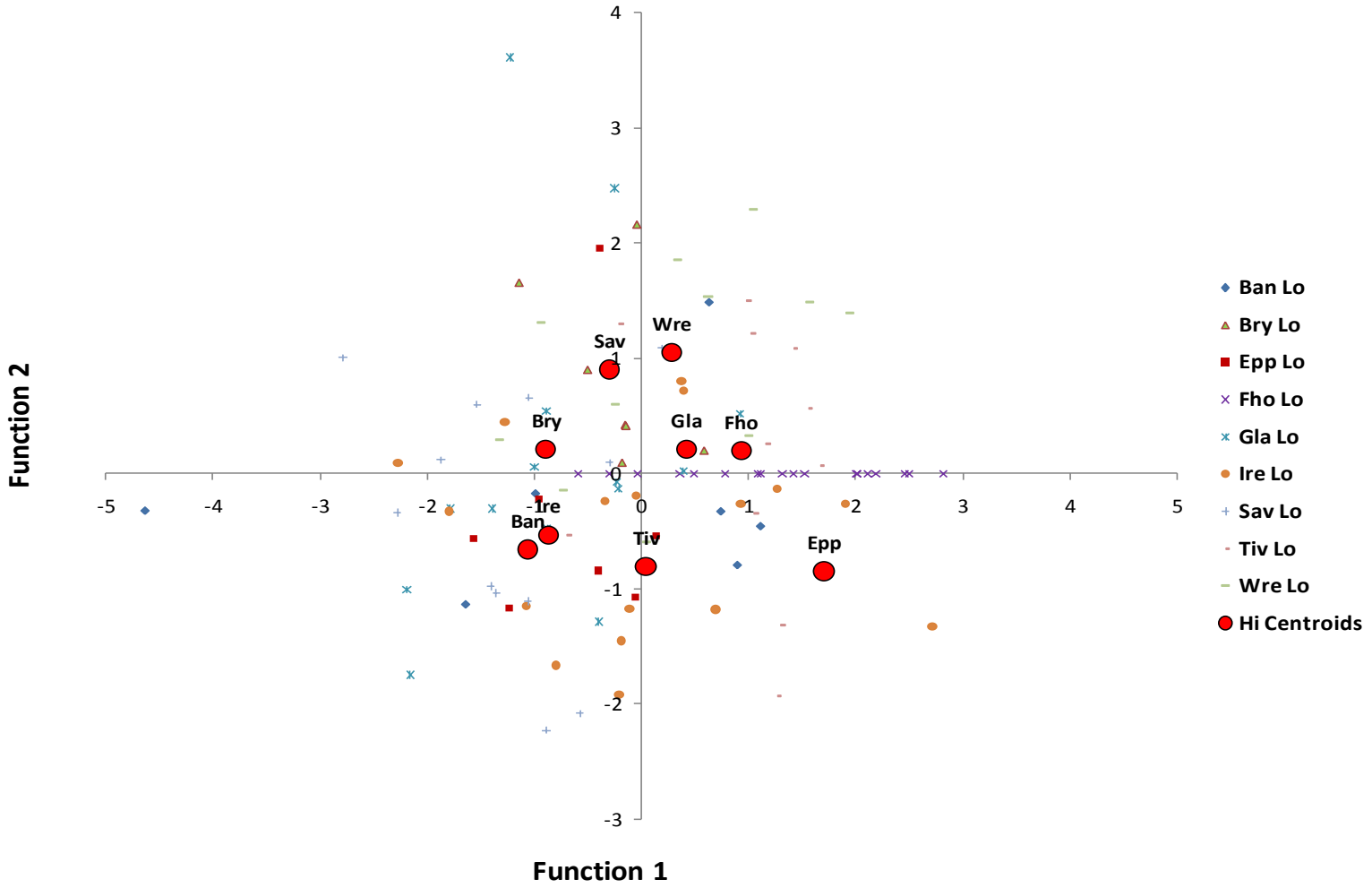


Figure 3.11. Results of discriminant analyses of high calcium populations in F_1 life history dataset. Coefficients for Function 1 and 2 are given in Table 3.16.

From Figure 3.11 the high calcium Epping population appears to stand out from the other populations, being characterised by higher positive values in function 1 and lower negative values in function 2. From table 3.17 it can be seen that when the high calcium populations are analysed alone, the weight to length ratio variable shifts from a strong positive correlation to a weak negative correlation in Function 1 (see Tables 3.14 and 3.17 respectively). This could suggest that the relationship between shell weight to length and other life history traits (e.g. age and length at first reproduction) differs in response to the availability of environmental calcium. The fact that a stronger correlation between weight to length ratio is seen in the low calcium group (see Table 3.14) would suggest that the relationship between shell weight and length and other life history traits becomes more detectable when populations are reared in low calcium media.

As age and length at first reproduction were strongly positively correlated with Function 1 (Table 3.17) this would suggest that of the high calcium populations, individuals from the Epping (and to a lesser extent Fauldhouse) populations would tend to take longer to reach reproduction which they do so at a larger size and with a lower weight to length ratio than the other populations. Conversely individuals in the Brychfa, Ireland and Bank populations would be expected to reproduce relatively earlier and at a smaller size when discriminated by Function 1.

With respect to Function 2 the Savernake and Wreake populations have higher positive scores and differ from the Bank Well, Ireland, Tiverton and Epping populations, which have lower negative scores on Function 2. In this instance the Savernake and Wreake populations would be expected to comprise of individuals that take a long time to reproduce with a higher weight to length ratio (i.e. heavier and/or shorter) than the other populations. The position of the Epping population centroid which displays high negative values (see Figure 3.11.) on the Function 2 axis suggests an apparent contradiction whereby this population is already characterised as having high age and length at first reproduction on the Function 1 axis but its position on the Function 2 axis suggests the contrary. As highest absolute values for age at

first reproduction and weight to length ration were found in Function 2 It is therefore perhaps best to consider the Function 2 as being most appropriate to separate these variables, the Epping population is best characterised as having individuals with a lower size at first reproduction and having a lower weight to length ratio.

Highest group classification was recorded in the Epping, Bank well and Savernake populations, with respectively 75%, 72.2% and 70% of individuals being correctly classified.

3.4.2.2. F_2 life history trait analysis via discriminant function analyses

Due to the fact that the Fauldhouse, Bank Well and Savernake populations had been retained for further study and were not subjected to culling after 21 days of reproduction, it was not possible to gather shell weight to length data as it had been for the other F_2 populations. As shell weight to length ratio had been proven to play a strong role in discriminating groups in the F_1 analysis discriminant analysis allowed 47.9% of the populations to be classified correctly and the results are displayed in Table 3.18 where it can be seen that 83.6% of the variation in the dataset could be explained by the first 3 functions. As before analysis focussed on the first 2 functions which accounted for 67.4% of the variance in the dataset.

Table 3.18. Results of discriminant analysis for life history traits, F_2 generation, high and low calcium with Savernake, Bank Well and Fauldhouse populations removed from analysis.

Function	Eigen value	Percent of variance	Cumulative Variance	Canonical correlation	Wilk's Lambda	Significance
1	1.258	42.1	42.1	0.746	0.198	<0.001, Y
2	0.756	25.3	67.4	0.656	0.243	<0.001, Y
3	0.486	16.2	83.6	0.572	0.426	<0.001, Y

Standardised discriminant coefficients for the first two functions are displayed in Table 3.19 while the structure matrix is displayed in Table 3.20. From Table 3.20 it can be seen that mean eggs per mass displayed the highest absolute negative correlation in the function 1. Age at first reproduction was shown to

display a strong positive contribution while next largest contribution came from the negatively correlated parameter *a*.

The second function displayed the largest absolute positive correlation between weight to length, length at first reproduction and parameter *a*. This indicates differences between these traits may be best observed in Function 2.

Table 3.19. Standardised canonical discriminant function coefficients for F_2 life history data with Savernake, Fauldhouse and Bank Well populations removed from analysis.

Life history trait	Function	
	1	2
Parameter *a*	0.050	-0.159
Total eggs in 21 days	0.237	-0.051
Mean eggs per mass	-0.942	-0.423
Mean survival to hatching	0.333	-0.292
Age 1st reproduction	0.483	-0.472
Length at first reproduction	-0.249	0.720
Weight to length	0.420	0.834

Table 3.20. Structure matrix displaying pooled within-group correlations between discriminating variables for first two functions with variables ordered by absolute size of correlation within function for F_2 life history data with Savernake, Fauldhouse and Bank Well populations removed. Asterisk (*) denotes largest absolute correlation between any variable and discriminant function while a indicates variables rejected from analysis.

Life history trait	Function	
	1	2
Mean eggs per mass	-0.640*	0.086
Parameter *b*[a]	0.301*	-0.086
Weight to length	0.191	0.690*
Length at 1st reproduction	-0.248	0.596*
Parameter *a*	-0.344	0.318*
Total eggs in 21 days	-0.264	0.142
Parameter *c*[a]	-0.248	0.081
Number of egg masses in 21 days[a]	0.297	0.050
Age 1st reproduction	0.538	-0.156
Mean survival to hatching	0.226	-0.124

The results of the discriminant analyses are displayed in Figure 3.12.

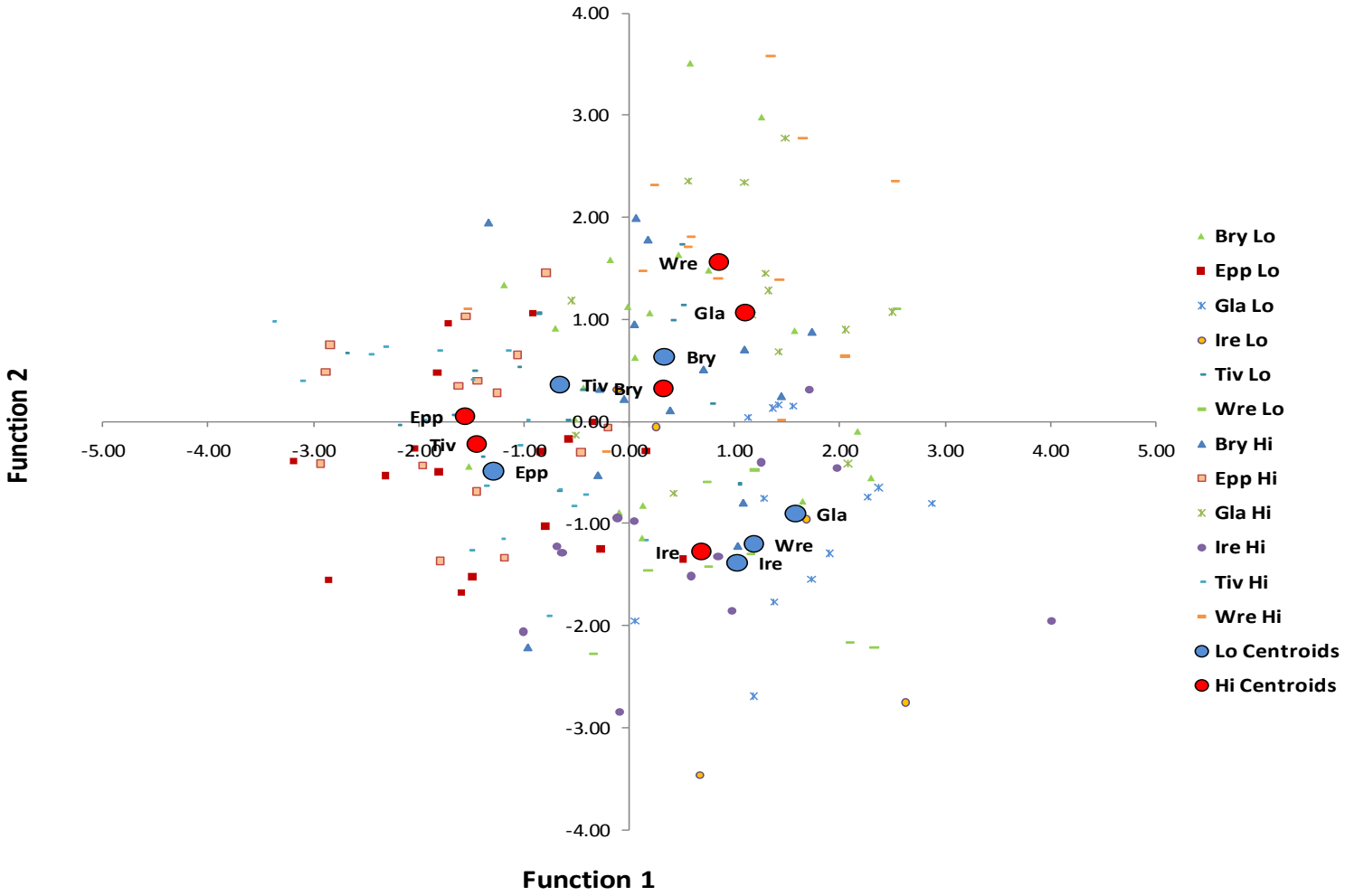


Figure 3.12. Results of discriminant analyses of F_2 life history dataset with nano-populations removed. Coefficients for Function 1 and 2 are given in Table 3.19.

From Figure 3.12. it can be seen that there is no clear separation of the high and low calcium groups as was seen in the F_1 analysis (see Figure 3.9.). On the Function 1 axis in Figure 3.12 the Glanahafren and Wreake populations score high positive values, while the high calcium Epping and Tiverton populations score high negative values.

As mean eggs per mass displayed the strongest (negative) correlation with Function 1 (Table 3.20) this would suggest that the Galanahafren and Wreake populations were characterised as those that produced less eggs per mass while the Epping and Tiverton populations produced more. As with the F_1 analysis age at first reproduction was found to have an important (positive) influence in discrimination on Function 1, indicating that the Glanahafren and Wreake populations would take longer to reach reproductive age, producing less egg per mass, while the Epping and Tiverton populations would reproduce sooner and produce more eggs per mass. This is suggestive of a trade-off between reproductive output and age of first reproduction however these variables are also likely to be influenced by other factors such as hatchling survival which was shown to be weakly positively correlated with Function 1 indicating that for populations displaying high negative values (such as Epping) that decrease in age at first reproduction was linked with reduced hatchling survival and increased reproductive output.

Weight to length, length at first reproduction and parameter *a* were found to have the largest absolute positive correlation with the discrimination in Function 2. In Figure 3.12 it appears that there is no clear overall separation of populations by calcium treatment. Some populations, such as Ireland and Epping and Brychfa, display very little separation across calcium treatment, remaining clustered together while the Wreake and and Glanahafren populations show a large shift in Function 2 scores between calcium treatments, whereby populations reared in low calcium have high positive Function 2 scores and those reared in low calcium have high negative Function 2 scores. This would suggest that in the F_2 generation the Glanahafren and Wreake populations responded to calcium treatment in a similar manner when reared in high calcium, by producing individuals that

could be characterised as having a high shell weight to length ratio (heavier per unit length), and taking a longer time to reproduce than when they were reared in low calcium media.

Highest group classification was recorded in the low calcium Wreake and Glanahafren populations and in the high calcium Tiverton population, with 88.9%, 61.5% and 61.1% of individuals respectively being classified correctly.

F_2 low calcium populations, discriminant function analyses:

Discriminant analysis allowed for 60.7% of all populations to be classified correctly (see Figure 3.13). The results of discriminant analysis are displayed in Table 3.21 where it can be seen that 100% of the variation in the dataset could be explained by the first 3 functions. As before analysis focussed on the first 2 functions which accounted for 95.7% of the variance in the dataset.

Table 3.21. Results of discriminant analysis for life history traits, F_2 generation, low calcium with Savernake, Bank Well and Fauldhouse populations removed from analysis.

Function	Eigen value	Percent of variance	Cumulative Variance	Canonical correlation	Wilk's Lambda	Significance
1	1.347	63.8	63.8	0.758	0.234	<0.001, Y
2	0.672	31.9	95.7	0.634	0.548	<0.001, Y
3	0.091	4.3	100	0.288	0.918	0.374, N

Standardised discriminant coefficients for the first two functions are displayed in Table 3.22 while the structure matrix is displayed in Table 3.23. From Table 3.23 it can be seen that mean eggs per mass makes the largest negative contribution to the first function while age at first reproduction makes a strong positive contribution followed by a weaker positive contribution from mean survival to hatching. Both mean eggs per mass and age at first reproduction were found to contribute the largest absolute correlation in Function 1, indicating that any observed population differences in this function are likely to be strongly linked to differences in these variables.

The second function displayed strong positive correlation between age at first reproduction and mean eggs per mass. A weaker negative correlation was shown between weight to length and Function 2.

Table 3.22. Standardised canonical discriminant function coefficients for F_2 low calcium treatments with Savernake, Fauldhouse and Bank Well populations removced from analysis.

Life history trait	Function	
	1	2
Mean eggs per mass	-0.767	0.872
Mean survival to hatching	0.498	0.141
Age 1st reproduction	0.489	0.812
Weight to length	0.063	-0.829

Table 3.23. Structure matrix displaying pooled within-group correlations between discriminating variables for first two functions with variables ordered by absolute size of correlation within function for low calcium populations F_2 life history data with Savernake, Fauldhouse and Bank Well populations removed. Asterisk (*) denotes largest absolute correlation between any variable and discriminant function while a indicates variables rejected from analysis.

Life history trait	Function	
	1	2
Mean eggs per mass	-0.723*	0.409
Age 1st reproduction	0.640*	0.437
Parameter *b*[a]	0.361*	-0.056
Parameter *a*[a]	-0.268	0.131
Parameter *c*[a]	-0.253*	0.151
Number of egg masses in 21 days[a]	0.244*	0.103
Mean survival to hatching	0.277	0.240
Weight to length	-0.086	-0.307
Total eggs in 21 days[a]	-0.326	0.276
Length at 1st reproduction[a]	-0.193	0.172

The results of the discriminant analyses are displayed in Figure 3.13.

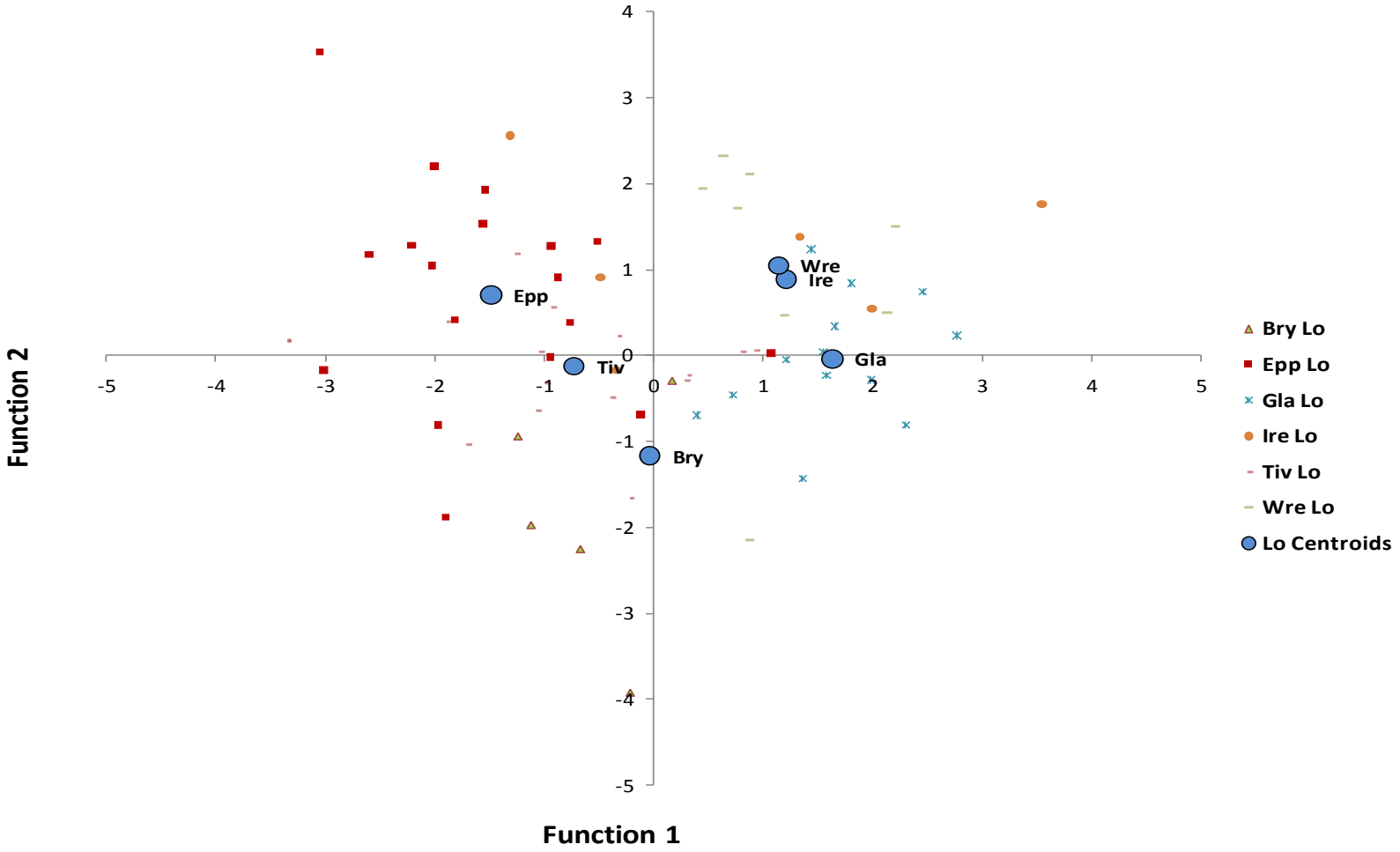


Figure 3.13. Results of discriminant analyses of low calcium populations in F_2 life history dataset with nano-populations removed. Coefficients for Function 1 and 2 are given in Table 3.22.

From Figure 3.13 there is clear separation of the different populations in the low calcium treatment. On the function 1 axis in Figure 3.13. the Wreake, Glanahafren and and Ireland populations score high positive values, while the Epping and (to a lesser degree) Tiverton populations score high negative values. On the function 2 axis the Wreake, Ireland and Epping populations display high popitive values and are distinguished from the Brychfa population which displays low negative values.

As with the high and low calcium analysis, mean eggs per mass displayed the strongest (negative) correlation with Function 1 (Table 3.23) this would suggest that the Galanahafren, Wreake and Ireland populations were characterised as those that produced less eggs per mass while the Epping and Tiverton populations produced more. Again age at first reproduction was found to have an important (positive) influence in discrimination on Function 1, indicating that the Glanahafren and Wreake populations would take longer to reach reproductive age, producing fewer eggs per mass while the Epping and Tiverton populations would reproduce sooner producing more eggs per mass. As with the high and low calcium analysis the weaker positive contribution of hatchling survival suggests a trade-off between survival and reproductive output i.e. individuals in the Glanahafren or Wreake populations take longer to reproduce, when they do they produce fewer eggs per mass but suffer low hatchling mortality.

Age at first reproduction and mean eggs per mass were found to have the largest positive correlation with the discrimination in Function 2, while weight to length was shown to have a negative correlation. On the Function 2 axis the Wreake, Ireland and Epping populations would therefore be considered to comprise of individuals that have higher age at first reproduction, greater eggs per mass and low weight to length ratios when compared with those in the Brychfa population. Consideration of Function 2 leads to an apparent trade-off between weight per unit length and age at first reproduction and reproductive output.

Highest group classification was recorded in the Epping, Glanahafren and Wreake populations, with respectively 72.2%, 69.2% and 66.7% of individuals classified correctly.

F_2 high calcium populations, discriminant function analyses:

Discriminant analysis allowed for 67.8% of all populations to be classified correctly (see Figure 3.14). The results of discriminant analysis are displayed in Table 3.24 where it can be seen that 97.6% of the variation in the dataset could be explained by the first 3 functions. As before analysis focussed on the first 2 functions which accounted for 78.3% of the variance in the dataset.

Table 3.24. Results of discriminant analysis for life history traits, F_2 generation, high calcium with Savernake, Bank Well and Fauldhouse populations removed from analysis.

Function	Eigen value	Percent of variance	Cumulative Variance	Canonical correlation	Wilk's Lambda	Significance
1	1.868	52.7	52.7	0.807	0.100	<0.001, Y
2	0.906	25.6	78.3	0.690	0.287	<0.001, Y
3	0.683	19.3	97.6	0.637	0.547	<0.001, Y

Standardised discriminant coefficients for the first two functions are displayed in Table 3.25 while the structure matrix is displayed in Table 3.26. From Table 3.26 it can be seen that the largest positive contribution to Function 1 comes from the weight to length variable, followed by age at first reproduction. As with the low calcium populations, mean eggs per mass was found to make a negative contribution to the first function. Weight to length was found to contribute the largest absolute correlation in Function 1, indicating that any observed population differences in this function are likely to be strongly linked to differences in this variables.

The second function displayed strong positive correlation between total eggs in 21 days, parameter *a*, and length at first reproduction, all three variables being found to have the largest absolute correlation with Function 2.

Table 3.25. Standardised canonical discriminant function coefficients for F_2 high calcium treatments with Savernake, Fauldhouse and Bank Well populations removced from analysis.

Life history trait	Function	
	1	2
Parameter *a*	0.007	0.260
Total eggs in 21 days	0.542	0.707
Mean eggs per mass	-1.038	-0.108
Mean survival to hatching	0.260	0.196
Age 1st reproduction	0.182	-0.369
Length at 1st reproduction	-0.223	0.272
Weight to length	0.970	0.042

Table 3.26. Structure matrix displaying pooled within-group correlations between discriminating variables for first two functions with variables ordered by absolute size of correlation within function for high calcium populations F_2 life history data with Savernake, Fauldhouse and Bank Well populations removed. Asterisk (*) denotes largest absolute correlation between any variable and discriminant function while a indicates variables rejected from analysis.

Life history trait	Function	
	1	2
Weight to length	0.611*	0.318
Total eggs in 21 days	0.030	0.793*
Parameter *a*	-0.063	0.704*
Length at 1st reproduction	0.115	0.693*
Parameter *c*[a]	-0.079	0.517*
Parameter *b*[a]	0.061	-0.421*
Mean survival to hatching	0.215	0.223
Mean eggs per mass	-0.284	0.587
Age 1st reproduction	0.365	-0.203
Number of egg masses in 21 days[a]	0.370	0.349

The results of the discriminant analyses are displayed in Figure 3.14.

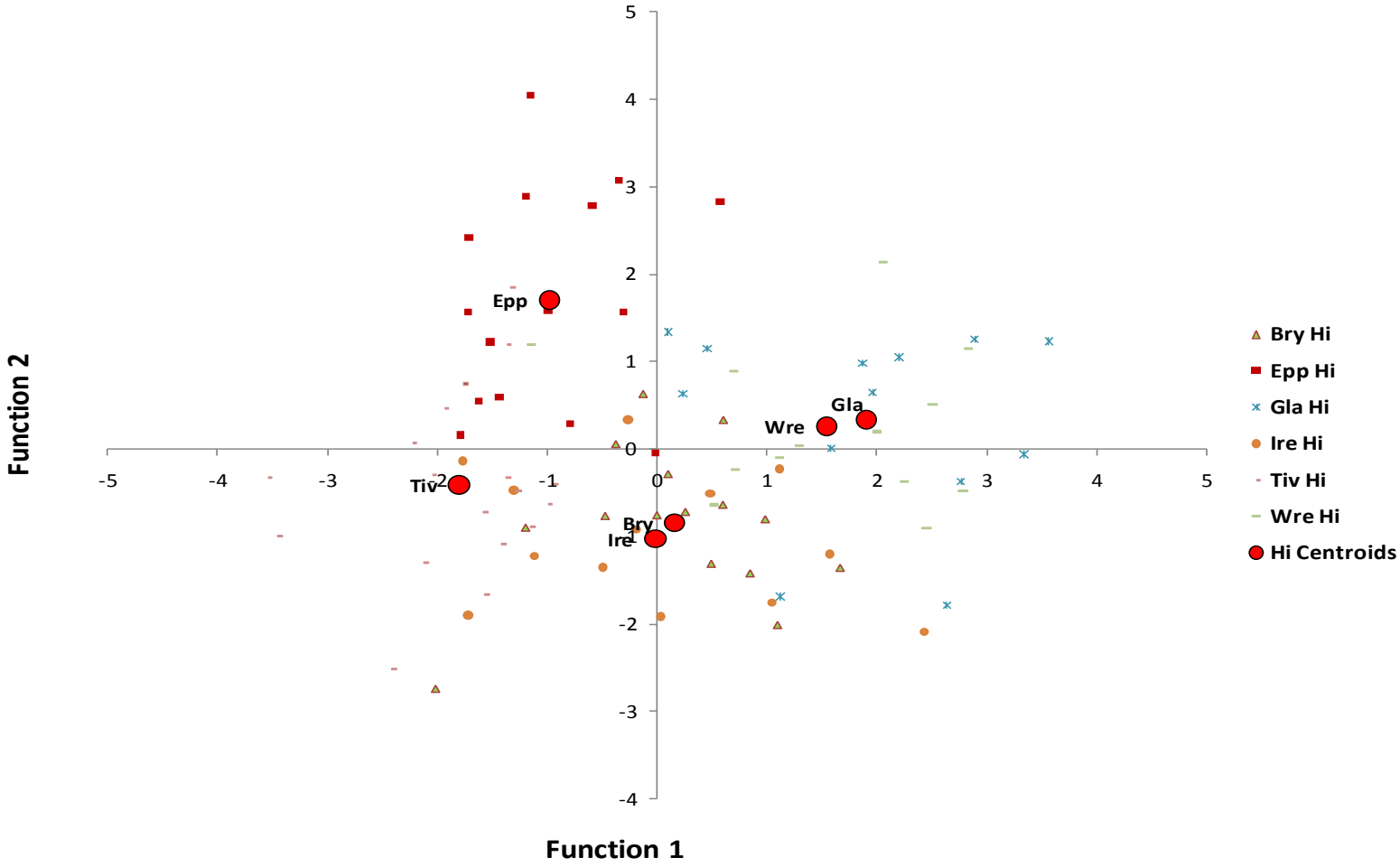


Figure 3.14. Results of discriminant analyses of high calcium populations in F_2 life history dataset with nano-populations removed. Coefficients for Function 1 and 2 are given in Table 3.25.

From Figure 3.14. it can be seen that there is clear separation of the different populations in the high calcium treatment. On the Function 1 axis in Figure 3.14 the Wreake, and Glanahafren populations score high positive values, while the Tiverton and (to a lesser degree) Epping populations score high negative values. On the Function 2 axis the Epping population displays high popitive values and is distinguished from the Brychfa and Ireland population which display negative values.

Weight to length ratio displayed the strongest (positive) correlation with Function 1 (Table 3.26). This would suggest that the Galanahafren and Wreake populations were characterised as those that had a high weight to length ratio (were heavier per unit length) while individuals from the Tiverton and Epping populations tended to be lighter per unit length. Again age at first reproduction was found to have an important (positive) influence in discrimination on Function 1, indicating that the Glanahafren and Wreake populations would take longer to reach reproductive age, producing fewer eggs per mass while the Epping and Tiverton populations would reproduce sooner producing more eggs per mass. Mean eggs per mass was again found to show negative correlation between Function 1 but was not found to be as important in discriminating between groups in the high calcium treatments.

Total Eggs in 21 days, parameter *a*, and length at first reproduction were found to have the largest positive correlation with the discrimination in Function 2. On the Function 2 axis the Epping population could therefore be considered to comprise of individuals that produce more eggs in 21 days, have higher size at first reproduction and ultimately attaining a larger size than individuals from the Brychfa and Ireland populations.

Highest group classification was recorded in the Tiverton, Ireland and Epping populations reared in high calcium, with respectively 83.3%, 76.9% and 73.3% of individuals classified correctly.

3.4.3. Matrix models

Lambda values

The F_1 and F_2 lambda values for the high and low calcium treatments for all populations are displayed in Figures 3.15 and 3.16 respectively. No significant difference in lambda was detected across either calcium treatment or population (Kruskall- Wallis Test, P>0.05).

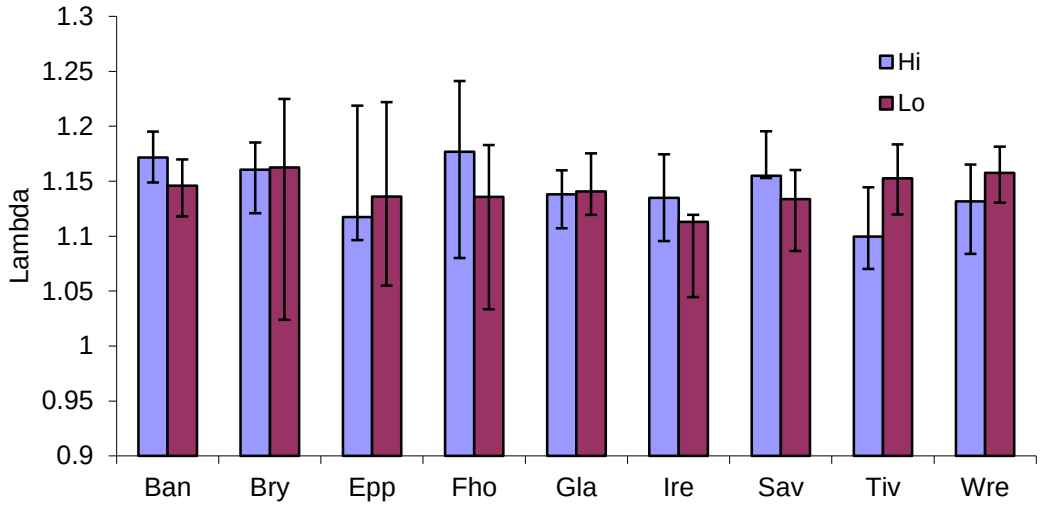


Figure 3.15. F_1 lambda values. Error bars denote 95% CI.

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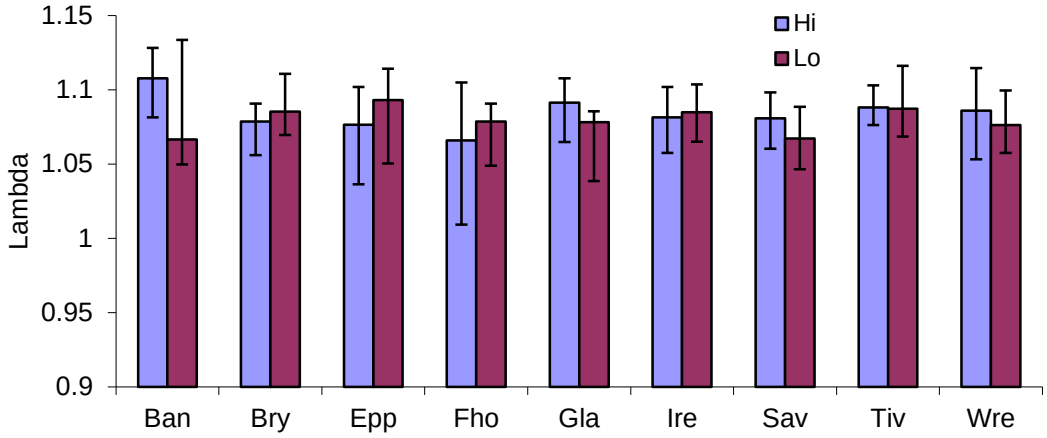

Figure 3.16. F_2 lambda values. Error bars denote 95% CI.

Mean lambda values for the F_1 and F_2 generation are displayed in Figure 3.17. No significant difference was found in mean lambda value across calcium treatment in the same generation (paired t-test, P>0.05), however a

significant decline in lambda was noted between the F_1 and F_2 generations (Paired t-test, F_1 mean = 1.14 ± S.E. 0.00476, F_2 mean = 1.08 ± S.E. 0.00298, DF=17, T= 10.51, P<0.001). When tested individually both the low (Paired t-test, F_1 mean = 1.14 ± S.E. 0.00498, F_2 mean = 1.07 ± S.E. 0.00443, DF=17, T= 8.67, P<0.001) and high calcium (Paired t-test, F_1 mean = 1.14 ± S.E. 0.00846, F_2 mean = 1.08 ± S.E. 0.00585, DF=17, T= 6.24, P<0.001) treatments displayed a significant decline in lambda across generations.

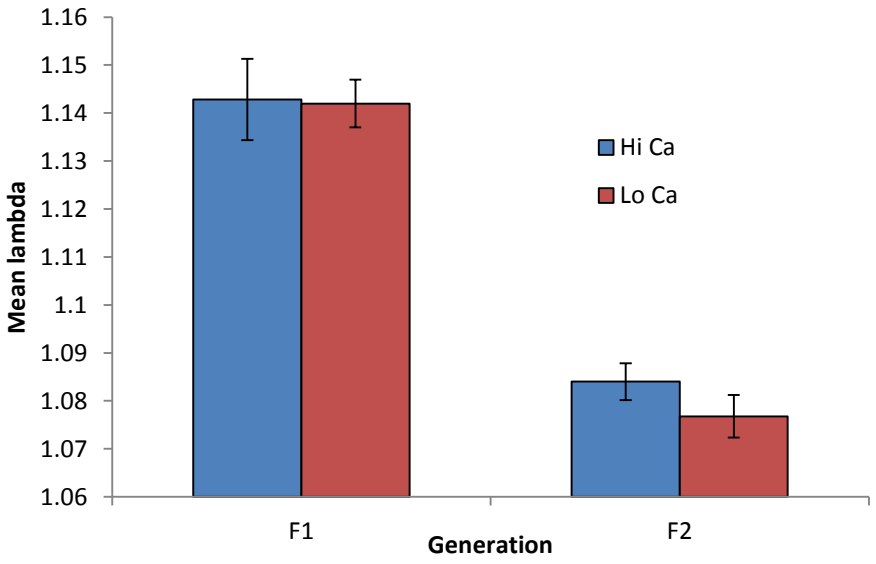

Figure 3.17. Mean F_1 and F_2 lambda values across calcium treatment. Error bars display standard error of mean.

The low calcium group displayed slightly greater difference in lambda across generations (Low calcium mean difference = 0.0652 ± S.E. 0.00752, High calcium mean difference = 0.0588 ± S.E. 0.00943) but this was found to not be significant (paired t-test DF=8, T=-0.53, P = 0.611).

Elasticity analysis

Elasticities for the F_1 and F_2 generations for all populations in high and low calcium treatments are displayed in Figures 3.18 and 3.19 respectively.

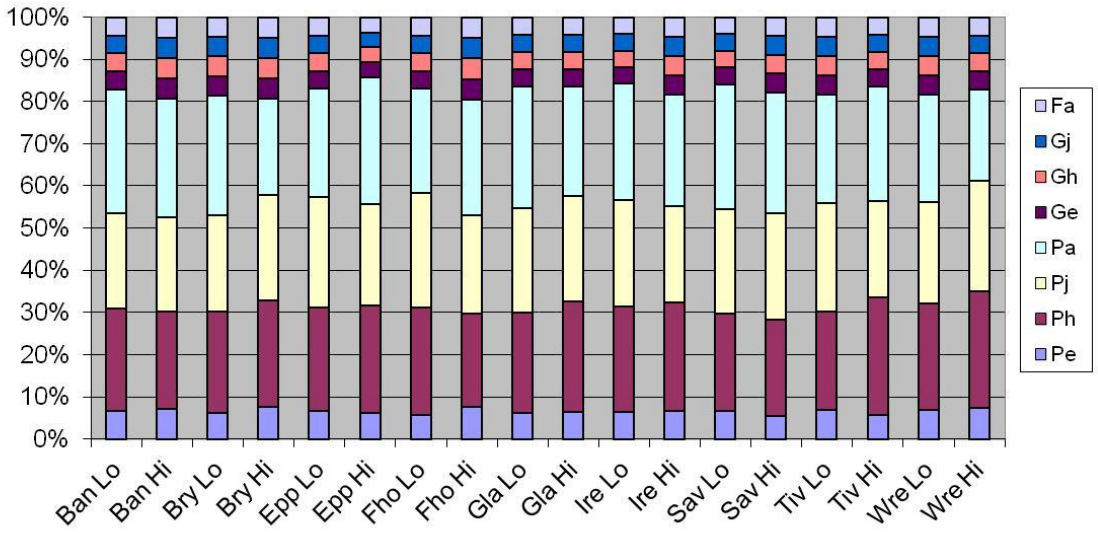

Figure 3.18. F_1 elasticity. Size of bar represents percentage contribution to lambda.

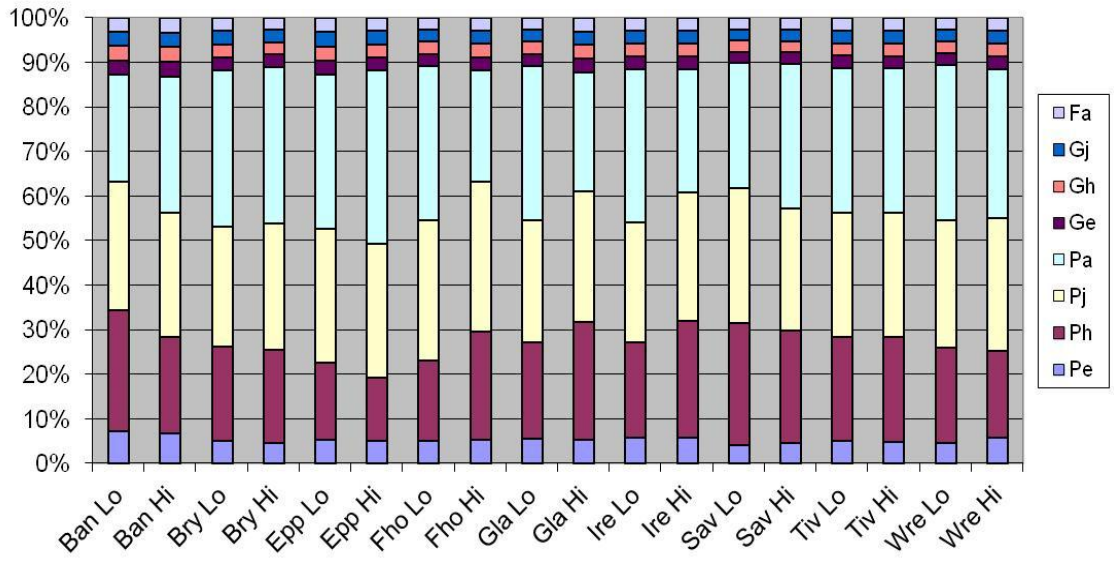

Figure 3.19. F_2 elasticity. Size of bar represents percentage contribution to lambda.

From figures 3.18 and 3.19 it can be seen that there are no clear consistent patterns in elasticities across populations or in response to calcium treatment in either the F_1 or F_2 generation. No significant differences were detected in any individual matrix parameter term at the population level or between calcium treatments in either the F_1 or F_2 generation (Kruskall Wallis Test $P>0.05$).

F_1 to F_2 linear comparisons

Elasticity data for each calcium treatment for both the F_1 and F_2 generations were compared using Spearman's rank correlation to assess the degree of similarity in percentage contribution to Lambda detectable between each treatment across generations. The output is displayed in table 3.27. Correlation coefficients were then compared to assess whether any differences in agreement between generations existed across calcium treatments (paired t-test, DF= 8, t = -2.08 P = 0.071). The results of the t-test indicate that there is no difference in correlation across generations by calcium treatment.

Table 3.27. Results of Pearson's correlation for F_1 vs. F_2 elasticity data.

Population	*Ca*	*n*	r_s	*P-value*	Significant
Ban	Lo	8	0.892	0.003	Y
Bry	Lo	8	0.973	<0.001	Y
Epp	Lo	8	0.973	<0.001	Y
Fho	Lo	8	1	<0.001	Y
Gla	Lo	8	1	<0.001	Y
Ire	Lo	8	1	<0.001	Y
Sav	Lo	8	1	<0.001	Y
Tiv	Lo	8	1	<0.001	Y
Wre	Lo	8	0.973	<0.001	Y
Ban	Hi	8	0.973	<0.001	Y
Bry	Hi	8	0.892	0.003	Y
Epp	Hi	8	0.973	<0.001	Y
Fho	Hi	8	0.973	<0.001	Y
Gla	Hi	8	0.892	0.003	Y
Ire	Hi	8	0.919	0.001	Y
Sav	Hi	8	1	<0.001	Y
Tiv	Hi	8	0.919	0.001	Y
Wre	Hi	8	0.892	0.003	Y

Mean percentage contribution to lambda for the F_1 and F_2 generations are displayed in Figures 3.20 and 3.21 respectively.

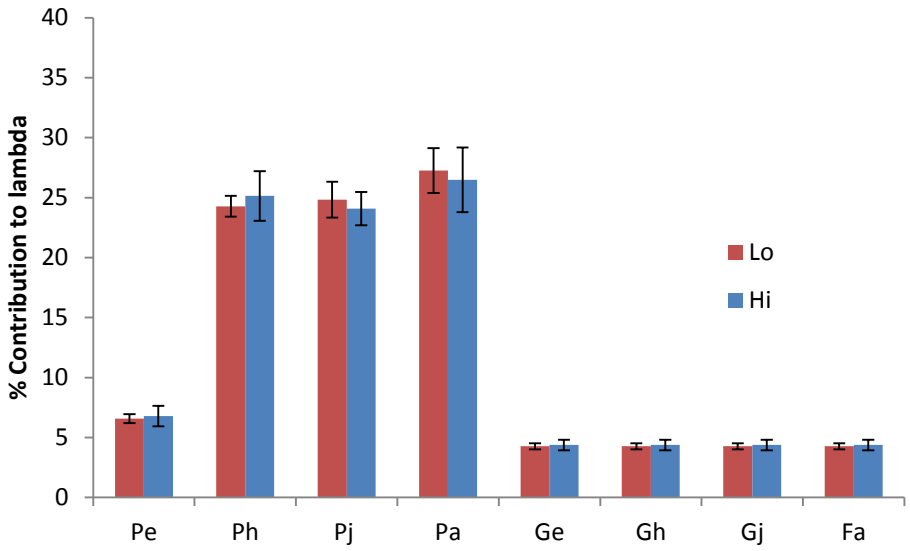

Figure 3.20. Mean F_1 percentage contribution to lambda by high and low calcium. Error bars show standard deviation.

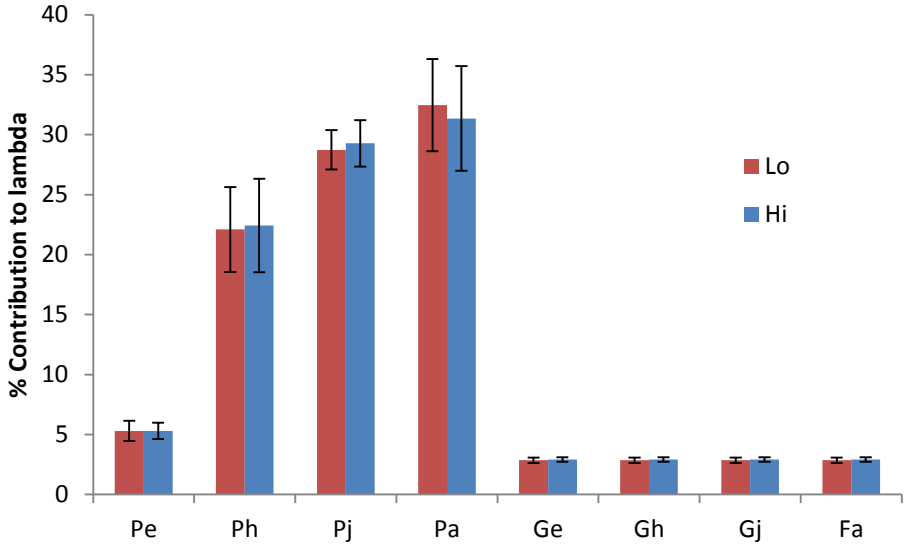

Figure 3.21. Mean F_2 percentage contribution to lambda by high and low calcium. Error bars show standard deviation.

From figures 3.20 and 3.21 it can be seen that the percentage contribution to lambda changes between the F_1 and F_2 generation. The mean percentage difference across generations in contribution to lambda for each calcium treatment is shown in Figure 3.22. Paired t-tests revealed that the differences across generations displayed in Figure 3.22 were significant for all parameter terms ($P<0.05$). The differences between the F_1 and F_2 generations are most marked by a shift in the percentage contribution from survivorship probabilities, P*i*. The adult survivorship term, Pa, was shown to contribute more to lambda in the F_2 generation, while the hatchling survivorship

probability, Ph, was shown to contribute less. This would indicate that hatchling mortality was greater in the F_2 generation, a likely result of inbreeding (Charlesworth and Charlesworth, 1987). Calcium treatment was not found to have any significant effects on contribution to lambda for any parameter term in either generation (paired t-test, P>0.05), although consistent trends towards higher contribution to lambda from Pa and less from Ph in the low calcium treatments are apparent in both generations (Figures 3.20, 3.21 and 3.22).

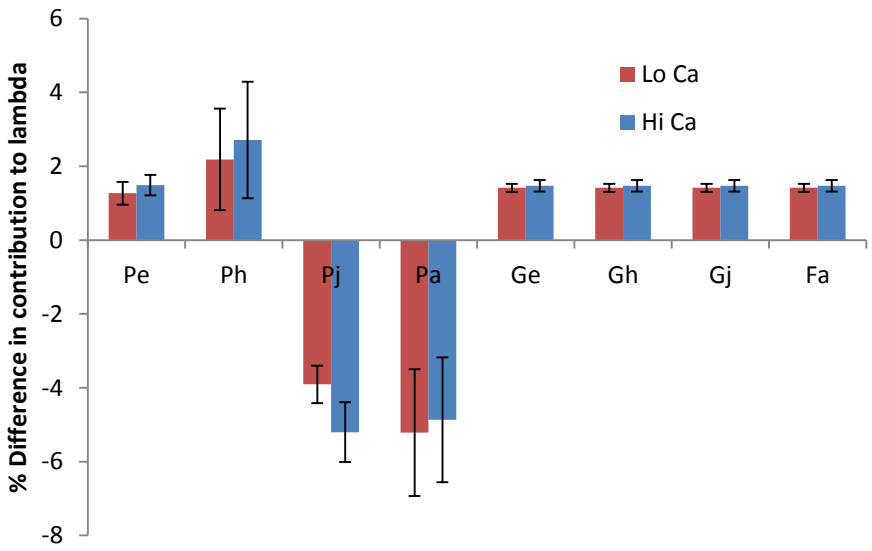

Figure 3.22. Mean differences in % contribution to lambda for each parameter term by calcium treatment F_1 – F_2 generation. Error bars denote standard error of mean.

Individual population differences in percentage contribution to lambda by high and low calcium treatment are displayed in Figures 3.23 and 3.24 respectively. When comparing Figures 3.23 and 3.24 it can be seen that the trend towards higher Pa values and lower Ph values in the F_2 generation becomes more consistent in the low calcium treatment groups (Figure 3.24), with more populations responding in a similar manner than in the high calcium treatment group (Figure 3.23).

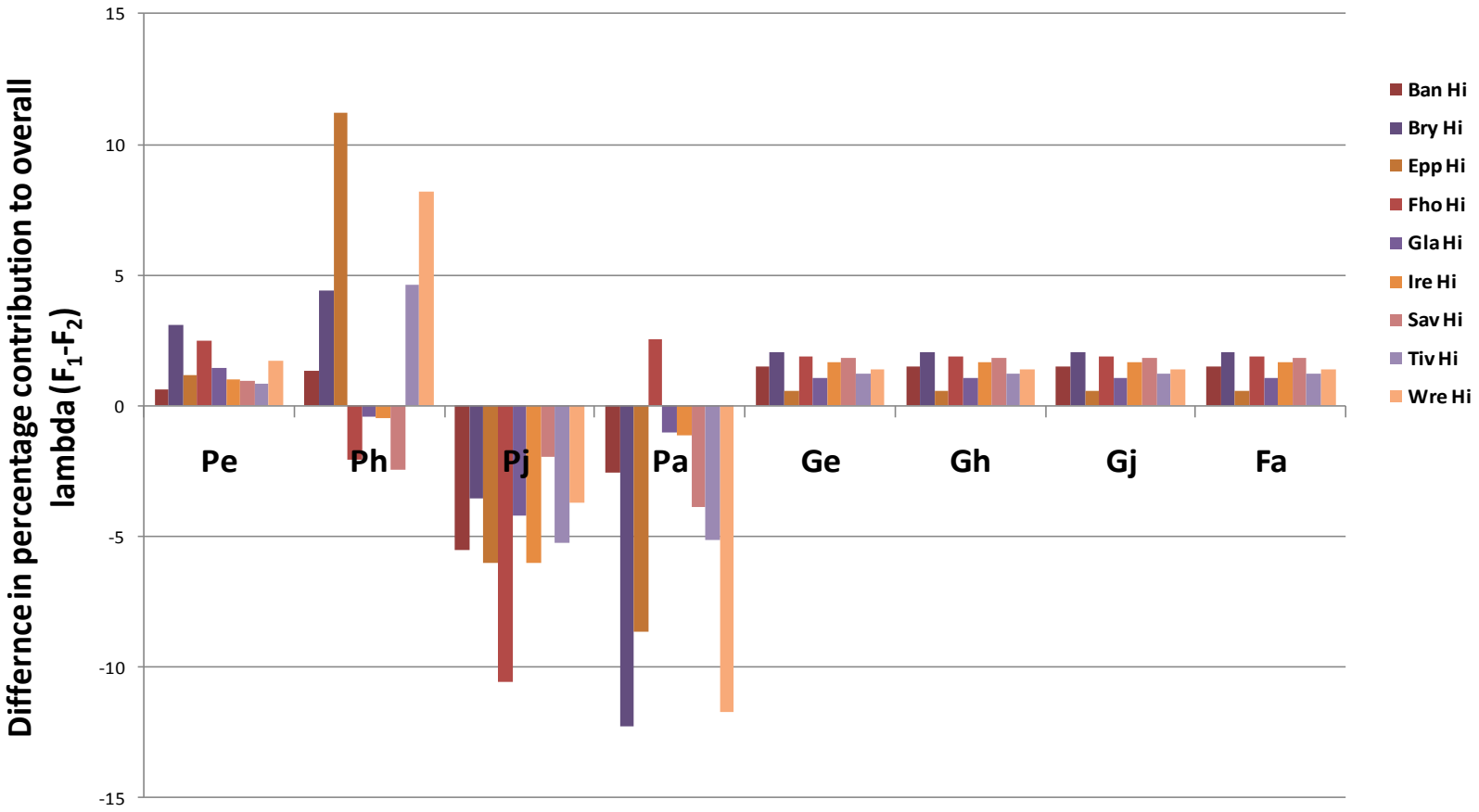

Figure 3.23. Difference in percentage contribution to overall λ value for each matrix parameter term between F_1 and F_2 generation for high calcium treatment. Positive values indicate that F_1 parameter term has greater importance in determining λ, while negative values indicate increased contribution to λ in the F_2 generation.

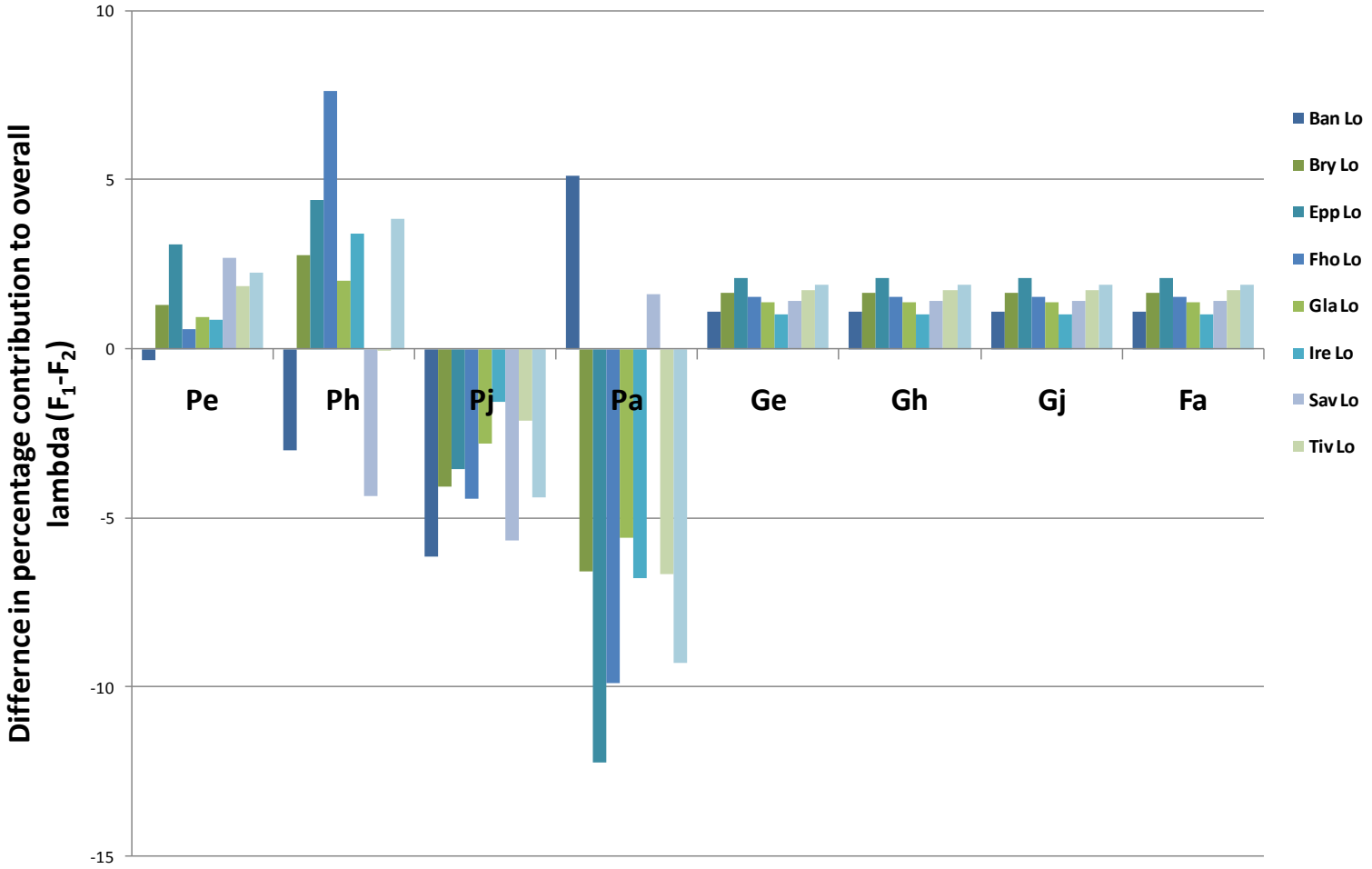

Figure 3.24. Difference in percentage contribution to overall λ value for each matrix parameter term between F_1 and F_2 generation for low calcium treatment. Positive values indicate that F_1 parameter term has greater importance in determining λ, while negative values indicate increased contribution to λ in the F_2 generation.

Individual population differences in percentage contribution to lambda across calcium treatment for the F_1 generation are displayed in Figure 3.25 and for the F_2 generation in Figure 3.26.

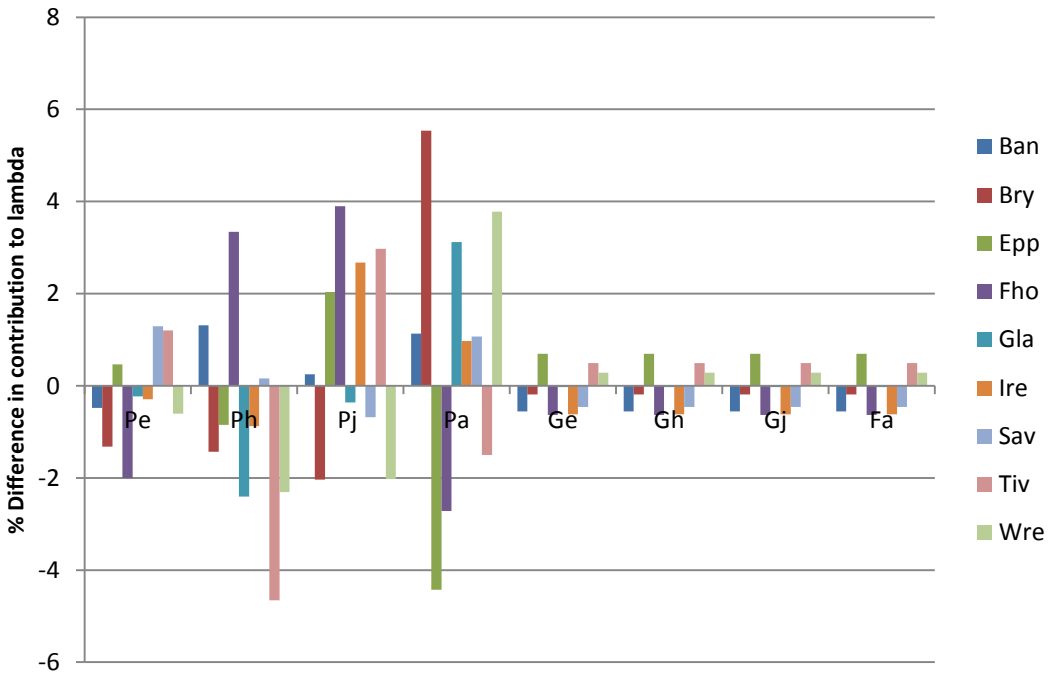

Figure 3.25. Difference in percentage contribution to overall λ value for each matrix parameter term between low and high calcium treatments for the F_1 generation. Positive values indicate that low calcium parameter term has greater importance in determining λ, while negative values indicate increased contribution to λ from the high calcium treatment group.

It would appear that the differences in percentage contribution to lambda across calcium treatments has increased between generations, with an approximately 10% range of difference in the F_1 generation increasing to an approximate range of 16% in the F_2 generation. The F_1 generation (Figure 3.25) displays a high degree of variability in response to calcium across populations with no clear pattern of response being observable in the study populations.

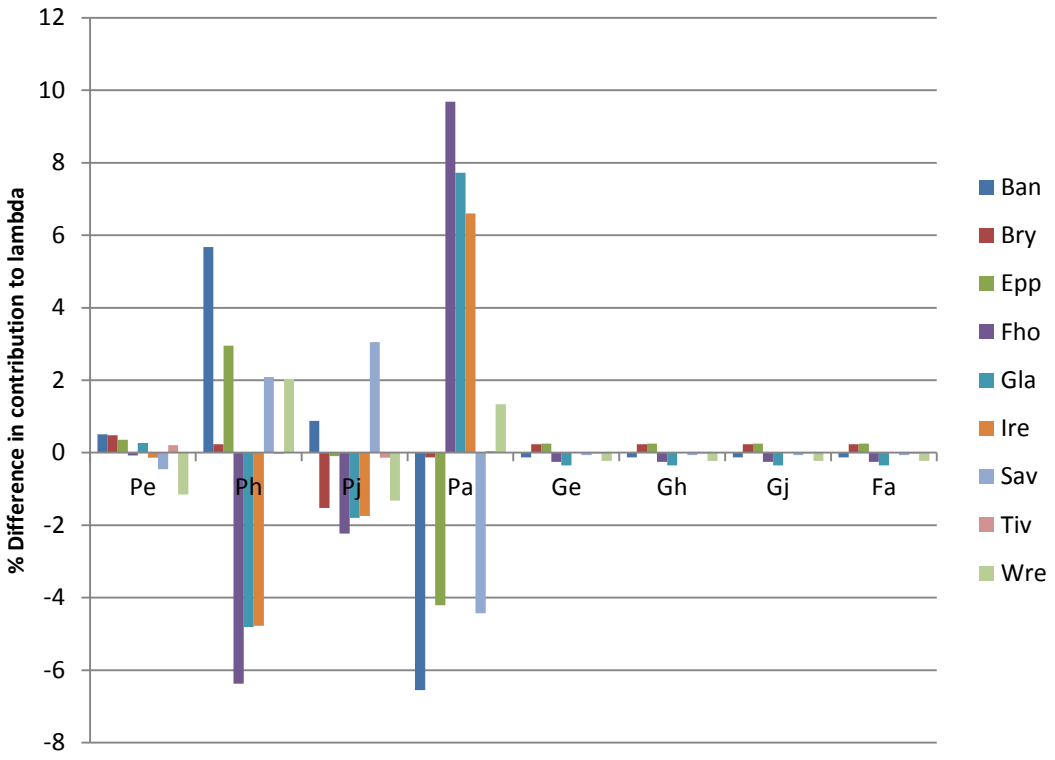

Figure 3.26. Difference in percentage contribution to overall λ value for each matrix parameter term between low and high calcium treatments for the F_2 generation. Positive values indicate that low calcium parameter term has greater importance in determining λ, while negative values indicate increased contribution to λ from the high calcium treatment group.

By the F_2 generation (Figure 3.26) it appears that more consistent patterns in response to calcium treatment have emerged. For example the Fauldhouse, Glanaharen and Ireland populations appear to display larger Pa and lower Ph values in the low calcium treatment, while the Bank, Epping and Savernake populations display the reverse trend with higher Pa values and lower Ph values in the high calcium treatment. Here the Fauldhouse, Glanahafren and Ireland populations appear to suffer higher hatchling mortality in the low calcium treatment (as represented by the increased contribution to the Pa term), while the Bank, Epping and Savernake population hatchlings appear to fare better in high calcium treatments.

3.5. Discussion

Trade-offs and relationships between traits

The PCA analysis revealed a generally consistent pattern of variation in the low and high calcium treatments across generations and the results of the PCA analysis are summarised in Table 3.28. In all analyses length at first reproduction was shown to correlate positively (as indicated by positive values in PCA loadings) with reproductive traits (Mean eggs per mass, total eggs and number of egg masses). A weak trade-off (where PCA loadings are shown to be opposed, see Figures 3.3, 3.4, 3.7 and 3.8) was observed between age and size at first reproduction (and correlated reproductive traits) in the low calcium F_1 generation. This relationship became more established in both calcium treatments in the F_2 generation but was still strongest in the low calcium treatment. This indicates that inter population differences between size and age at reproduction are more evident when environmental stress (in the form of reduced calcium availability) is high. This is in keeping with the view that different strategies in response to environmental stress have evolved in different populations.

As reproductive traits are correlated with size at first reproduction, it would also appear that early reproduction in this study resulted in reduced reproductive output associated with smaller sizes, while later reproduction was associated with larger individuals and greater reproductive outputs. Numerous studies on other species have demonstrated that trade-offs between age and size at first reproduction typically result in variation in offspring size and number, where smaller more numerous offspring are associated with smaller, earlier reproduction and fewer, larger offspring result from delayed reproduction at a larger size (Reznick, 1983, Reznick et al., 1990, Roff et al., 2002, Stearns, 1983).

While egg size was not measured in this study, reproductive output in numbers was shown to directly correlate with adult size and it appears that the trade-off between age and size at first reproduction does not result in the classic trade-off described above. One likely reason that this is not observed in *L. stagnalis* is due to physiological constraints imposed by the challenges of

Table 3.28. Summary findings of PCA analysis investigating chief trade-offs between life history traits (Figures 3.3, 3.4, 3.7 and 3.8) -, --, --- indicates increasing degree of negative association between traits while +, ++, +++ indicates increasing positive association. Strongest trade-offs are highlighted in bold. The PC where separation/association is observed is displayed, as separation by the first component is likely to indicate a stronger relationship than by PC2. Trade-offs between the separate reproductive traits are considered below.

Trade-off	*F_1 Low Ca*		*F_1 High Ca*		*F_2 Low Ca*		*F_2 High Ca*	
	PC1	*PC2*	*PC1*	*PC2*	*PC1*	*PC2*	*PC1*	*PC2*
Age at 1st reproduction Vs. Total eggs in 21 days	**--**	**--**	**+**	**--**	**---**		**--**	**-**
Length at 1st reproduction Vs. Total eggs in 21 days	**+**	**-**	**++**	**-**	**+++**	**-**	**+++**	
Age at 1st reproduction Vs. Length at first reproduction	**-**		**+**		**---**	**-**	**--**	
Survival to Hatchling Vs. Total eggs in 21 days	**--**		**+**	**--**	**+**		**+**	
Trade-offs within reproduction:								
Mean eggs per mass Vs. Number of egg masses in 21 days		**-**			**+**	**---**		**---**

living in fluctuating freshwater environments, whereby in order to cope with variable abiotic conditions, developmental stages have been telescoped resulting in a larger egg (McMahon, 1983). Such physiological constraints on egg size appear to be conserved across the majority of freshwater mollusc species and may explain why the trade-off between size and age at first reproduction is not as pronounced in freshwater molluscs as it is in other genera (Dillon, 2000).

Age at first reproduction was shown to display a weak association with reproductive traits, confirming as reported in the literature, that size rather than age at maturity appears to be a better predictor of reproductive output in freshwater gastropods (Dillon, 2000).

These results indicate a strong association between size at reproduction and reproductive output across generations. This suggests that size at reproduction is under strong genetic control but that age at maturity (and therefore growth rates) show considerable plasticity in the face of varying environmental conditions (calcium availability). These findings support those in the literature that suggest that either one of size or age at first reproduction tend to be fixed while the other trait remains variable (Stearns, 1992). For example a study by Ford and Seigel (1994) demonstrated that age at first reproduction in the snake, *Elaphe guttata*, could be significantly altered by diet yet size at maturity remained constant. Similar findings in this study suggest that size at maturity is fixed in *L. stagnalis* and that environmental calcium is analogous to diet in the above study causing variation in age at maturity.

Reproductive traits were shown to show little separation between populations in the F_1 generation while greater population separation was observed in the F_2 generation, suggesting that removal of environmental influence allowed trade-offs in reproductive traits to be revealed. A weak trade-off between the number of eggs per mass and the number of egg masses was apparent in the low calcium group in the F_1 generation and became strongly established in both treatment groups by the F_2 generation. This suggests that those populations that tended to lay fewer egg masses laid larger ones while those

that laid more tended to lay smaller ones and reflects different strategies employed across populations. Within the general strategy of iteroparity, this may represent alternative patterns of reproductive effort which may be related to the survival of eggs and juvenile stages in the natal environment, which is also known to strongly influence overall reproductive strategy (Stearns, 1992), with multiple, smaller egg masses selected for in more variable environments (Calow, 1978, McMahon, 1983). The fact that the trade-off between reproductive traits was only weakly detected in the low calcium group in the F_1 generation and then subsequently detected in both treatments in the F_2 generation, would suggest that this trade-off is only in part affected by environmental calcium, and once environmental background is removed, the underlying genetic basis of this relationship becomes more apparent. Additional support for this view comes from the high heritabilities of reproductive traits and size at maturity reported in Chapter 2. These results indicate that local adaptation of life history in *L. stagnalis* in this study appears to be strongly defined by variation in reproductive traits (and subsequent trade-offs therein) and associated size at first reproduction.

It is worth noting that due to the nature of the dataset used not all life history traits recorded in the study were able to be analysed via PCA and some significant trade-offs may have not been detected in this analysis. For example other than egg survivorship this method takes no consideration of age specific mortality schedules which may trade-off against reproduction (Begon et al., 2006) and, due to co-linearity with size at first reproduction, growth parameter terms were omitted from the PCA. A further possible trade-off between egg size and number might have been detected if egg size had been measured but this was not deemed practical due to time constraints. Defence for this omission comes from the fact that this relationship has not tended to be as strongly defined in freshwater gastropods as it is in other genera (Dillon, 2000).

Intra-specific variation in life histories

The principle findings of the discriminant analyses are summarised in Table 3.29. The discriminant analyses revealed that traits that allowed best

Table 3.29. Summary findings of discriminant function analysis investigating which trait best separates between populations and calcium treatment.

Treatment	*Dominant trait(s) and loading (+, -)*		*Interpretation/comments*
	Function 1	***Function 2***	
F_1 Complete dataset	Length 1st reproduction (+) Age 1st reproduction (+)	Length 1st reproduction (+) Weight to length (+)	Separation by calcium in Function 1. Low calcium Fauldhouse population larger and older at 1st reproduction
F_1 Low calcium	Length 1st reproduction (+) Weight to length (+)	Parameter *b* (+) Age 1st reproduction (+)	Strongest separation in Function 1 Fauldhouse population relatively larger with heavier shells.
F_1 High calcium	Age 1st reproduction (+) Length 1st reproduction (+)	Length 1st reproduction (+) Weight to length (+)	Strongest separation in Function 1 Epping population takes longer to reach 1st reproduction at larger size. Bank, Ireland and Brychfa reproduce early at smaller size.
F_2 Complete dataset	Mean eggs per mass (-) Age 1st reproduction (+)	Weight to length Length at 1st reproduction	Most separation by Population in Function 1 Wreake, Glanahafren and Ireland populations produce fewer eggs per mass and reproduce later while Epping and Tiverton populations produce more eggs per mass and reproduce earlier.
F_2 Low calcium	Mean eggs per mass (-) Age 1st reproduction (+)	Age 1st reproduction (+) Mean eggs per mass (+)	Strongest separation in Function 1 Wreake, Glanahafren and Ireland populations produce fewer eggs per mass and reproduce later while Epping and Tiverton populations produce more eggs per mass and reproduce earlier
F_2 High calcium	Weight to length (+) Age 1st reproduction (+)	Total eggs in 21 days (+) Parameter *a* (+) Mean eggs per mass (+)	Strongest separation in Function 1 Glanahafen and Wreake populations are relatively heavier and reproduce later while Epping and Ireland populations are lighter and reproduce earlier

separation between populations and calcium treatments changed between generations. In the F_1 analyses length at first reproduction was shown to consistently deliver a strong contribution to the first discriminant function, along with weight to length ratio. The strong contribution of variation in length at first reproduction is consistent with the high degree of variation in this trait evident from the PCA analysis. In contrast, the first discriminant function in the F_2 analyses consistently separated groups by age at first reproduction and eggs per mass. The removal of three populations from the F_2 analysis due to use for the nano exposure experiments (Chapter 4) complicates interpretation of differences between F_1 and F_2 generations, but the increased emphasis on age at first reproduction rather than length, along with reproductive effort in the form of eggs per mass could suggest that these traits are more plastic/susceptible to environmental variation, whereas size shows a more consistent pattern of variation across generations, particularly in low calcium treatments (Chapter 2) and hence is more likely to be under genetic control.

Response to calcium stress:

F_1 generation

The F_1 discriminant analysis of all populations combined displayed clear separation between high and low calcium on Function 1 (Figure 3.9). Separation between groups was derived from differences in length and to a lesser extent age at first reproduction and from weight to length ratios, whereby the low calcium populations were distinguished from the high calcium groups by taking longer to reach a reproductive size and having relatively lighter shells.

When analysed individually both the low and high calcium groups were best separated by differences in length and age at first reproduction (Figures 3.10 and 3.11). However, populations responded differently across calcium treatments. In the low calcium analysis the Fauldhouse population was shown to be distinguished from others by taking longer to reach a larger reproductive size with a higher weight to length ratio while the Epping population was similarly distinguished in the high calcium analysis. Weight to length ratios were shown to have strong positive correlation with Function 1 in

the low calcium group but a weak negative association was displayed in the high calcium group. This suggests that inter population differences in weight to length ratio are more detectable when snails are reared in low calcium as would be expected when this resource is more limited (Stearns, 1992).

F_2 generation

The F_2 discriminant analysis revealed no clear separation across calcium treatments when all populations were analysed (Figure 3.12). Age at first reproduction and number of eggs per mass were found to be most important in separating groups. Again the Epping population was shown to be distinct from others being characterised as having a high number of eggs per mass and a lower age at first reproduction relative to the other populations. Populations were shown to cluster together when separated by Function 1 suggesting that genetic differences rather than response to calcium treatment were becoming more important in discriminating between groups in the F_2 generation. It would appear that by the F_2 generation the observed separation between groups in Function 1 results primarily from population level effects. From Figure 3.12 low calcium populations tended to score consistently higher on discriminant function 1 relative to their high calcium counterparts suggesting that while effects of environmental calcium on life history traits may be subtle and vary in intensity between populations, they operate in a generally conserved manner across populations. Results would indicate that low calcium populations were separated on the grounds as being those that tended to reproduce later and produce fewer eggs per mass than their high calcium counterparts. Highest group separation in response to calcium was observed in discriminant Function 2, but was shown to be population specific with some populations displaying little to no response. The Wreake and Glanahafren populations show the greatest response across calcium treatments and were shown to separate on the basis of higher weight to length ratios, lengths at first reproduction and larger absolute sizes when reared in high calcium.

Individual analysis of the low and high calcium treatments (Figures 3.13 and 3.14) revealed that the same principal traits, namely age at first reproduction

and eggs per mass were important in separating groups. Differences across calcium treatment came from the fact that weight to length ratio was most important in the high calcium group displaying the reverse trend that was displayed in the F_1 generation. Populations appeared to display some consistency of response across calcium treatments with the Epping population being characterised as having greater eggs per mass and reproducing faster than other populations while the Wreake and Glanahafren populations reproduced later and produced fewer eggs per mass. In the high calcium treatment population differences were also defined by variation in shell weight to length ratios, whereby shells from the Epping population tended to be relatively lighter than those from the Glanahafren and Wreake populations. It is possible that this could represent a trade-off between shell weight and reproductive output whereby investment in one trait dictates the allocation of resources to another. Differences in shell weights are known to be linked to risk from predation (Krist, 2002, Lewis and Magnuson, 1999) so the observed pattern could be linked to variation in predator pressures (such as crayfish) at the different sites, which were not assessed in the initial sampling.

The patterns of variation in terms of the traits that have been identified as best at classifying populations and calcium treatments through the discriminant function analysis are to some extent consistent with the key sources of variation shown in the PCA analysis, particularly in the F_1 generation. However in the F_2 generation there is some shift in the key traits identified, which was not evident in the broad patterns of variation summarised in the PCA analysis of the F_2 generation. This would tend to suggest that it is not always the traits that show the broadest patterns of variation that are the most important in distinguishing populations or the effects of calcium availability. Some traits may show consistently high variation between individuals, but no characteristic variation between populations or in response to calcium treatment, with such differences instead driven by more subtle patterns of variation not always evident in a PCA analysis. Further, observed differences may have causal links to other traits. For example size rather than age at reproduction is known to dictate maturity in freshwater molluscs (Dillon, 2000)

and one would expect populations to be discriminated more by size at reproduction rather than age. In this analysis size at first reproduction was found to best separate groups in the F_1 generation, however by the F_2 generation groups tended to be best separated by age at first reproduction. One possible explanation for this trend could be that age at maturity is more plastic than size in response to environmental variation. Here inter population differences in size at maturity are relatively fixed and are met by variation in age at maturity in response to environmental conditions, causing differences in size at maturity to be eclipsed in this type of analysis. Data on growth and age at reproduction presented in Chapter 2 and the results of the PCA analysis above support the view that greater plasticity is found in traits associated with growth (and therefore age at first reproduction), while inter population differences in size at first reproduction were more conserved across generations. This would indicate that there are causal linkages between traits that need to be considered when using this type of analysis. Similarly this argument could also be extended to the increased importance of eggs per mass in discriminating between groups in the F_2 analysis whereby this trait appeared to display more variation across calcium treatments and populations (and therefore provided better separation of groups analysed) than other reproductive traits which appeared to display lower heritabilities (Chapter 2, Figures 2.62-2.64).

Conclusions drawn from the results of the discriminant function analysis are contingent on the fact that in all cases, the success of prediction was not particularly high, being around 50% in most cases. This would again suggest that in most cases, variation in life history traits and trade-offs is not strong in the populations studied, although there may be individual exceptions to this such as the Fauldhouse and to a lesser extent the Epping populations which seem to show a greater degree of distinction in life history. In the F_1 generation high calcium treatment the Epping population took longer to reproduce at a larger size with a lower weight to length ratio than the other populations. The Epping population was again found to be distinct from other populations in the F_2 generation but was discriminated by different traits, being characterised as producing more eggs per mass in both calcium

treatments and reproducing sooner than other populations in the low calcium treatment, while as with the F_1 study this population tended to produce relatively lighter shells than the other populations in the high calcium treatments. Similarly the F_1 Fauldhouse population took longer to reproduce at a larger size than the other populations when reared in low calcium and displayed a greater shell weight to length ratio than the other populations. Such differences could be driven by local environmental factors such as temperature (Yampolsky and Scheiner, 1996) or predation (Lewis and Magnuson, 1999) or may simply be the result of strong founder effects as this population was known to have been introduced to the area.

A study by Brown (1983) used discriminant analysis to examine variation in life history traits at different levels (between families, populations and habitat types). The patterns of variation that were evident in this study broadly reflect the findings of Brown (1983) in so far as it was size at maturity and clutch size that were the most consistent traits that separated the groups analysed. It would appear that similar characteristics that can be used to analyse and classify patterns of variation in life history across a broad sweep of freshwater mollusc species (Brown, 1983, Calow, 1978, Dillon, 2000) are also the key traits that separate individual populations of a species as in this study. Given the nature of the speciation process these findings are not entirely surprising.

Population level response in life history to environmental stress

Matrix models allowed the responses of different life stages to high and low calcium to be combined and compared at the population level via the calculation of the intrinsic population growth rate, lambda. The resultant lambda values indicate that calcium concentration has little effect on population growth rate. No differences in lambda across population or calcium treatment were recorded in either generation with no consistent pattern of variation in response to calcium treatment being evident. Lambda values for populations in high and low calcium for both generations were shown to be above 1, indicating that all populations were displaying positive population growth. A significant decline in lambda was observed between generations and was likely to be a result of inbreeding depression due to the

animals being forced to self (Charlesworth and Charlesworth, 1987, Coutellec-Vreto et al., 1998). While not significant the mean low calcium F_2 lambda value was shown to be lower than the mean high calcium value suggesting a trend towards reduced population growth in low calcium populations. Even a small difference in lambda could have profound implications toward population growth when combined with other environmental stressors such as pollution and predation (Sandrine et al., 2009). It is possible that the calcium values used in this study were not sufficiently low enough to challenge the organisms and that had lower calcium concentrations been used then a greater reduction in lambdas may have been recorded in the low calcium populations.

Elasticity analysis revealed no differences in any matrix parameter term at either the population or treatment level for either generation. Significant differences in percentage contribution to lambda were recorded in all parameter terms across generations (Figure 3.22). These differences were defined by the F_2 generation displaying an increased percentage contribution to lambda from the adult and juvenile survivorship parameters (Pa and Pj), and a reduction from hatchling survivorship (Ph) and fecundity (Fa). This would suggest that the effects of inbreeding across generations resulted in a higher overall contribution to population growth being derived from the adult and juvenile life stages via a reduction in hatchling survivorship and fecundity. This is consistent with a study by Coutellec-Vreto et al. (1998) who suggested that increased hatchling mortality was associated with purging of lethal mutations in *Lymnaea peregra.* While not significant the differences in contribution to lambda across generations in the low calcium treatment displayed a larger contribution from the Pa and lower contribution from Ph and Pj than the high calcium group. This would suggest that in general populations in the F_2 generation reared in low calcium tended to suffer greater juvenile and hatchling mortality than those in high calcium resulting in a higher contribution to lambda from the Pa term. The individual population differences in contribution to lambda across generations by high and low calcium treatment displayed in Figures 3.23 and 3.24 reveal a decomposition of this effect across populations. It can be seen that a more conserved

response towards a greater contribution to lambda in Pa from the F_2 generation is apparent in the low calcium group (Figure 3.24), with the strongest contributions coming from the Epping, Fauldhouse and Tiverton populations. Similarly a conserved reduction in contribution to lambda in Ph is noted in the F_2 low calcium populations, with the Epping and Fauldhouse populations showing the strongest reduction in Ph across generations.

The maximum range of difference in percentage contribution to lambda across calcium treatment was shown to increase across generations by more than 50% from approximately 10% to 16% (Figures 3.25 and 3.26). This would indicate that population differences in response to calcium became more pronounced in the F_2 generation when environmental effects had been bred out. The F_1 generation displays no strong pattern of variation across calcium treatments. By contrast the F_2 generation can be more easily separated into those populations in which Pa contributed more and Ph contributed less in contribution to lambda in the low calcium groups (Fauldhouse, Glanahafren and Ireland), and those where Pa contributed more and Ph contributed less in the high calcium groups (Bank, Epping and Savernake). This would indicate that different populations respond differently to the same environmental stressor. In this study the F_2 Fauldhouse, Glanahafren and Ireland populations displayed higher early life stage mortality in the low calcium treatment while the Bank, Epping and Savernake populations appeared to display the reverse trend with greater early mortality in the high calcium treatments. The remaining Brychfa, Tiverton and Wreake populations displayed little response at the population level to calcium.

Such subtle differences in the percentage contribution of parameter terms to lambda across calcium treatment discussed here do not appear to show any consistent patterns in determining overall lambda values. For example in the F_2 study both Epping and Fauldhouse populations had a slightly higher lambda value in the low calcium treatment despite contrasting contributions from the Pa and Pj parameter terms.

3.6. Conclusion

Size at first reproduction was shown to display a strong positive correlation with reproductive traits, whereby larger individuals were shown to produce a greater number of offspring. Age at first reproduction was found to be less strongly associated with reproductive traits in the F_1 analysis and became negatively correlated with reproductive traits and size at first reproduction by the F_2 generation. A trade-off between age and size at first reproduction was apparent across both calcium regimes. The results of the PCA suggest that age at first reproduction is more plastic than size which appears to be under stronger genetic control as supported by the high heritability displayed in inter generational comparisons. A further reproductive trade-off between number of egg masses and eggs per mass became established in both calcium treatments by the F_2 generation.

In the discriminant analyses different traits delivered the best separation between generations. Removal of environmental differences in the F_2 generation suggest that observed differences may be driven by fixed population specific differences in size at first reproduction, whereby plasticity is observed in traits related to growth such as age at first reproduction in response to different levels of environmental stress and less so in traits with a more direct link to fitness such as size at first reproduction. A similar case is possibly seen in eggs per mass whereby other reproductive traits such as egg number and number of egg masses display higher heritabilities across generations.

A significant decrease in population growth rate between the F_1 and F_2 generations was likely the result of inbreeding due to selfing. Inbreeding significantly decreased hatchling survivorship and fecundity causing the adult stage to contribute more to lambda in the F_2 generation. Calcium was found to have no significant effect on population growth rate although tentative evidence suggests that lambda was slightly lower in the F_2 low calcium treatment. Elasticity analysis revealed a general trend towards greater contribution from adult stages towards lambda in the low calcium groups. Calcium stress appears to induce the same effects on population growth rate

(albeit at a much smaller scale) as inbreeding (Coutellec-Vreto et al., 1998) and would suggest that a shift towards adult dominance in contribution to lambda may be a common response to stress.

4. Effects of nanoparticle exposure on *Lymnaea stagnalis*

4.1. Abstract

The effects of exposure to carbon black nanoparticles on different life cycle stages of the great pond snail, *Lymnaea stagnalis* were investigated. The extent of population differences, along with the influence of environmental variation in the form of calcium availability, on observed responses to carbon black exposure were also examined. Acute exposure to carbon black was found to significantly affect growth, survivorship, feeding, and reproduction, with earlier life stages after hatching displaying the most profound effects. Individual stage exposures revealed significant differences in mortality and growth between both populations and calcium treatment at the hatchling level and a significant effect on growth at the juvenile level. Adult animals did not display any response either in terms of growth or mortality. Data from the individual stage exposures were used to derive inputs for a stage-classified matrix model which was then used to derive population growth rates (λ). Populations reared in high calcium were all found to display positive population growth rates (λ above 1) while lower λ values were found in populations reared in the low calcium media. Populations differed in the extent to which population growth rate was influenced by calcium treatment. Elasticity analysis revealed that hatchling survivorship was most affected by combined stressors. However, the extent of the response differed between populations, suggesting that responses to carbon black exposure and potentially other toxicants are influenced by both the environmental context and differences between populations.

4.2. Introduction

The development and production of nanoparticles (NPs; materials with three dimensions under 100 nm in size, across a range of application areas has increased very rapidly in recent years (Klaine et al., 2008). Alongside this development concerns have been voiced regarding the potential impact of nanoparticles in the environment (Moore, 2006, Klaine et al., 2008). These concerns include potential direct toxicity, persistence in the environment and biological systems, as well as potential for food chain uptake (Oberdorster et al., 2005, Wiesner et al., 2006, Maynard et al., 2006). Studies in mammalian systems have established that exposures to nanoparticles can lead to increased production of reactive oxygen species (Stone et al., 1998) which may lead to oxidative stress (Stone et al., 1998) and inflammation (Brown et al., 2001). However, it is well known that uptake, clearance and any effects of nanoparticles depend on a variety of factors, such as, constituent material, surface coating, surface charge, media type, pH, organic matter and redox conditions (Handy et al., 2008).

Despite the increased production of nanomaterials (NMs) in recent years, studies on their potential effects on species other than humans are still relatively limited (Colvin, 2004), although the range of aquatic organisms that have been studied has expanded considerably in recent years, and now covers photosynthetic taxa such as algae and seaweeds (Nielsen et al., 2008, Ji et al., 2011), invertebrates (Asghari et al., 2012, Lovern and Klaper, 2006, Lovern et al., 2007, Rosenkranz et al., 2009, Musee et al., 2010, Pradhan et al., 2012) and vertebrates (Gaiser et al., 2012, Zhu et al., 2008).

In aquatic systems, which are highly likely to be subject to unintentional releases of nanoparticles through wastewater and other routes (Klaine et al., 2008) there is a rapidly growing literature on effects on organisms at the individual level (Gaiser et al., 2012, Glenn et al., 2012, Lovern and Klaper, 2006, Musee et al., 2010, Nielsen et al., 2008, Oberdorster et al., 2006, Pradhan et al., 2012, Rosenkranz et al., 2009). Releases of NMs potentially effect a wider range of organisms within aquatic food webs. However, given the tendency of NMs to aggregate/agglomerate (Moore, 2006) and thus not

easily suspend in aquatic media, organisms that live on or among the benthos are also likely to be exposed, and potentially more so than planktonic taxa. Scaling up from current data and information on individual responses to population or higher level effects is not straightforward for several reasons. Firstly, while many studies have found that nanoparticulate forms of relatively non-toxic parent material such as carbon black (CB) and titanium dioxide (TiO_2) are more toxic than their equivalent non-nanoparticulate forms (Brown et al., 2001, Nielsen et al., 2008) the concentrations required to result in observed toxic effects in test subjects in individual-based toxicity assays often have greatly exceeded that likely to be realistically released into the environment (Gottschalk et al., 2009, Nowack, 2009) and thus cannot easily be extrapolated to likely effects at the population level. Secondly, most studies of contaminant effects, not just of nanoparticles, are based on one population or clone of laboratory-reared organisms, yet there is increasing evidence to show that different populations or genotypes of organisms vary in their responses to stressors through local genetic adaptation (Lopes et al., 2006). Finally, laboratory-based assays are generally taken out of any environmental context, often through the use of standard media. Again such practices are beneficial for reproducibility of results, but natural populations are subject to a wide range of environmental conditions across their geographical ranges (Gaston, 2003) resulting in local adaptation of life histories and other characteristics (Lam and Calow, 1989a, Jensen et al., 2008, Brown, 1983) which influence the response of populations to contaminants (Paul-Pont et al., 2010, van Ooik and Rantala, 2010, Duchet et al., 2010, Miaud et al., 2011).

Here the aim is to redress the balance to some extent by focusing on a benthic grazing organism, *Lymnaea stagnalis,* and assessing the effects of acute and chronic exposures to carbon black (CB) nanoparticles under varying environmental conditions (in the form of calcium availability) before considering effects at the population level via the use of matrix population models.

Acute exposure studies involved exposing different life stages of *L. stagnalis* to a range of carbon black (CB) (14.3 nm) nanoparticle concentrations for 21 days and effects on mortality, growth, feeding, reproduction and physiological condition (Fat, and combined carbohydrate (CHO) and protein content) were recorded. Chronic exposure studies were subsequently conducted to examine how different populations of *L. stagnalis* responded to low-level CB exposure under high and low calcium availability; calcium being a fundamental nutrient required in the construction of the shell (Dillon, 2000) which is known to vary greatly across the range of this species (Kerney, 1999). Three different populations (from different areas of the UK) and life cycle stages (hatchling, juvenile and adult) of *L. stagnalis*, were exposed to carbon black NP and effects on growth, mortality and reproduction were recorded over a 42 day period. The data obtained from the experiments were used to parameterise a stage-classified matrix model to predict the responses of the different populations and examine changes in the contributions of different life stages to overall population growth rate. Matrix models are widely used to scale up from individual to population responses to contaminants (Hodgson and Townley, 2004, Iwasaki et al., 2010, Klok et al., 2007, Forbes et al., 2008), but only rarely have responses of different populations or strains been compared (Salice and Miller, 2003, Forbes et al., 2001). The key aim of this part of the study, rather than establish the impact of carbon black nanoparticles *per se*, was rather to assess effect of environmental conditions (low and high calcium concentration) on the response shown by different life stages and overall population growth rate, and how this varied between three different UK populations of *Lymnaea stagnalis*.

4.3. Materials and methods

4.3.1. Nanoparticle preparation and characterisation

Particle preparation

All solutions were prepared by stepwise dilution from stocks using Artificial Pond Water (APW) (ASTM, 1980) adjusted to provide a calcium concentration

of 200mgL^{-1}. Stock solutions of 1000mgL^{-1} were prepared using ultrafine carbon black (Degussa Printex Furnace Black, 14.3nm) and weighed accurately in a glove box prior to being sonicated in an ultrasound bath to ensure complete suspension prior to stepwise tenfold dilution to 0.01mgL^{-1} which included the concentrations used in this study. Each step in dilution required that the media be sonicated (bath sonicator, 35 kHz frequency, Kerry PUL325) for 30 minutes to provide complete suspension of the carbon black in the media. Media were then decanted into exposure vials/cups for 24h and allowed to settle prior to animals being introduced as it was surmised that this procedure was more representative of the natural fate of CB.

Particle characterisation

Characterisation of the CB nanoparticles was performed using a ZetaSizer Nano ZS (Malvern Instruments Inc, UK) where the hydrodynamic size and the surface charge (zeta potential) of nanoparticle dispersions were obtained utilizing dynamic light scattering (DLS) and electrophoretic light scattering (ELS), respectively. Suspensions were prepared by stepwise dilution of 1gL^{-1} CB (Printex Furnace Black - 14.3nm diameter) in appropriate media (see below) to yield solutions of 1mgL^{-1} CB in 40 and 200mgL^{-1} calcium APW. Following preparation, the medium was filtered through a 0.2 micron filter before the particles were added and the suspension sonicated in an ultrasonic bath (Cole Parmer, 8893, USA, 135W, 42KHz ± 6Hz) for 1hour. Subsamples (10ml) were immediately taken via pipette and transferred to sample vials. Characterisation was performed straight after this transfer and also after settlement had taken place for 24 hours. In both cases the supernatant was run on the instrument, to reflect more accurately the conditions of the experiments (see below). Three repeated measurements were taken for each calcium treatment and settling regime and both mean diameter and zeta potential were recorded, to allow for assessment and comparison of particle agglomerate/aggregate size as well as dispersion stability within each suspension and time interval (Stone et al., 2010).

4.3.2. Direct acute exposure of *Lymnaea stagnalis* to carbon black NP

Embryos and juveniles in this study were derived from the Union Canal in Edinburgh (NT236720) while, due to insufficient adult snails being present in the Union Canal, the adults were taken from Fauldhouse pond in West Lothian (for details of location see Chapter 2). All stock populations were held in APW (ASTM, 1980) adjusted to a calcium concentration of 200mgL^{-1}. All experimental treatments were housed in the controlled environment chamber at 17^{o}C with a 12h: 12h light: dark cycle.

4.3.2.1. Embryo exposure

Three individual egg masses from the Union Canal population were harvested and cut into pieces using a sterilised scalpel, before being placed in a range of CB concentrations (0.1, 1.0, and 10 mgL^{-1}) and controls in sample vials. Five replicates were used per treatment. Three egg masses in all were used the first two being large enough to split between two replicates each (i.e. cut into eight), while the last mass was used only in the fifth replicate. This yielded sections of egg mass containing approximately 15 eggs in each exposure vial. Previous studies indicate that cutting the masses does not affect embryo survival (Wagner, 2000). The number of live embryos in each segment was determined by observing movement under a light microscope. The media were changed twice weekly and at each change the number of embryos surviving was counted and photographs of each segment taken to determine growth rates. Length measurements were taken from five randomly selected embryos from each treatment after 5 weeks of exposure, with length being defined as the longest measurement across the embryo. The experiment ran for 40 days.

4.3.2.2. Juvenile exposure

Individual juvenile snails from the Union Canal population, hatched 8 days prior to exposure were exposed to 10, 1.0, 0.1 and 0.01mgL^{-1} of CB and controls in 30ml of test solution/control media in 125cm^3 PTFE jars. The choice of this concentration range was based on studies of different

organisms (Nielsen et al., 2008, Rosenkranz et al., 2009), as well as pilot studies. Five replicates at each concentration were prepared. The snails were photographed under a dissecting microscope at x20 magnification to allow size and subsequent growth to be determined before being placed in the exposure jars. The snails were fed *ad libitum* on iceberg lettuce. Media were exchanged twice weekly with any mortality recorded at these points. The experiment was allowed to run for 5 weeks. Photographs of each individual were taken at each change of media to determine any effects on growth. Length measurement was defined as the distance from the anterior to the furthest point away on the spire of the shell (see Figure 4.1).

Figure 4.1. Length measurement on juvenile *Lstagnalis*.

4.3.2.3. Adult exposure

Individual adult snails from Fauldhouse pond were exposed to 250cm^3 of 100, 10 and 1mgL^{-1} CB and controls in large plastic tubs. Ten adult snails were selected for each treatment and control. Initial lengths were recorded using vernier callipers and the snails were fed iceberg lettuce *ad libitum*. Adult feeding was determined by soaking discs of iceberg lettuce in media for 1h (to absorb as much water as possible) and then taking initial weights before being placed in the exposure tub with the snail for 24h. At the end of this period the remaining lettuce was surface dried and weighed to allow the food consumed to be determined. This procedure was carried out once a week for the three weeks of the adult exposure. The adults were also measured

weekly, as described in 4.2.2.2., to determine whether any treatment effect on growth could be found.

Adult reproduction was monitored throughout the experiment. The number of egg masses was recorded and the number of eggs per mass was determined by analysis of images derived from a digital microscope camera.

4.3.2.4. Movement analysis

After the 21 days exposure, all surviving adults were filmed using a digital camera for a period of 1 min to determine whether any differences in displacement could be determined. Animals were placed in a shallow dish of water of sufficient depth to cover the snail completely, allowed to attach to the substrate and settle for a short period (judged to be once the snail was moving). A light source was then placed above the top end of the tray to provide a directional impetus to movement and the snails were then filmed for a period of 1 minute. Still frames captured from the video were then used (using the front of the foot as a reference point) to derive start and end coordinates, allowing total displacement to be calculated using Pythagoras' theorem.

4.3.2.5. Fat, protein and carbohydrate content.

After 21 days the adult fat content was determined by ether extraction as described by Reznick (1983). This involved removal of the viscera from the shell by blanching in boiling water for <5 seconds to release it from the shell. Once removed the viscera were oven dried at 60^{o}C to constant mass (mass 1) and placed in anhydrous ether to extract lipids. Ether extraction was carried out until no further colour change in the ether could be observed by the naked eye. The viscera were then allowed to air dry before being placed in the oven again at 60^{o}C and reweighed (mass 2). Thereafter the viscera were ashed in a muffle furnace at 550^{o}C and reweighed yielding mass 3. Mass 1 minus mass 2 yielded the total lipid (in the form of triglycerides) content while the difference between mass 2 and 3 yields the protein and carbohydrate (CHO) content of the tissues.

4.3.3. Intra specific variation in chronic CB exposure effects under different environmental conditions

Experiments were based on three selected populations of the F_2 adult laboratory generation or their progeny described in Chapter 2. Snails were reared in isolation in porous 200ml plastic cups in the flow-through system contained within the controlled environment facility at 17^{o}C with a 12H:12H dark:light regime and fed iceberg lettuce *ad libitum* until they reached a size suitable for use in experiments (see below for details). The media contained within the flow-through systems was based on Artificial Pond Water (APW) (ASTM, 1980) modified to give calcium concentrations of 40 (low calcium) or 200 (high calcium) mgL^{-1} (See table 2.2) These values were chosen to represent contrasting levels of calcium within the variation experienced across the UK geographic range (see Chapter 2). Egg masses obtained from the field surveyed adults and their subsequent progeny were maintained in separate systems at either high or low calcium levels for the two generations prior to the experiments being performed.

4.3.3.1. Population selection

Three populations described in Chapter 2 were selected for further study: Fauldhouse in Southern Scotland (National Grid Reference NS623910), Bank Well in Lancashire (SD471754) and Savernake in Southern England (SU221651). The Fauldhouse population is an introduced population, beyond the natural UK northerly range limit, and was established prior to 1970 (Kerney, 1999) although the date and source are unknown. The other two populations were, as far as is known, natural populations. Selection was based on the responses across the F_1 and F_2 experiments (see Chapter 2) and aimed to select those populations which had displayed distinctive life histories and/or strong responses in life history traits across calcium treatments.

4.3.3.2. Exposure of different life cycle stages

Three different life stages were selected for exposure: hatchlings, mean size (±SD) at start of exposures 2.13mm (±0.33); juveniles (pre-reproductive individuals), mean size (±SD) at start of exposures 8.20mm (±1.97); and adults (reproductive individuals), mean size (±SD) at start of exposures 27.27mm (±3.34).

Exposures were performed in 30mL of 1mgL^{-1} CB in either high or low calcium APW in 200mL plastic cups. Snails were fed iceberg lettuce *ad libitum* throughout the experiments and the media were changed once weekly. Individual lengths were measured prior to the exposure period (either via vernier callipers or via digital photographs, depending on the size of individuals) and every two weeks subsequently until the 42 day exposure period ended. Reproduction (which could only occur via selfing as individuals were isolated) was recorded by removing and photographing any egg masses found within the cups to determine the number of eggs laid.

4.3.4. Population level effects

Matrix model development

A stage-classified matrix model was constructed as described in section 3.2.3. Four life cycle stages were defined for model development: eggs, hatchlings, juveniles and adults (see Figure 3.1) and the stages were defined and analysed as described in section 3.2.3. Pilot studies showed that egg hatching time and success were unaffected by exposure to CB at the concentration used, so the data used in the models were derived from the F_2 generation which was reared under the same environmental conditions but in the absence of CB nanoparticles.

Hatchlings and juveniles were analysed separately in the experiments and in the model on the basis that previous observations had shown that mortality was relatively high in the period immediately after hatching, but reduced as individuals grew larger. The hatchling stage was considered to last from hatching until when individuals reached the mean initial size of individuals

used in the juvenile exposure group. The juvenile stage was considered to last from the end of the hatchling stage to the time when individuals reached the mean initial size of individuals used in the adult exposure group. In both cases, the time taken for individuals to reach the start of the next stage was estimated by linear extrapolation of growth data obtained from the experimental work.

The four life cycle stages are represented in the life history diagram displayed in Figure 3.1. This diagram was then used to create the projection matrix, *A* (3.1), where the P_i, G_i and F_i values correspond to those in Figure 3.1. The model had a time step of one week and was constructed and analysed according to the methods and equations described for stage classified matrix construction by Caswell (2000).

4.3.5. Statistical analyses

All statistical analyses were performed using either SPSS V.15 or Minitab V.15 for Windows. In the acute and chronic tests mortality was assessed using Kaplan-Meier non-parametric survivorship analysis with censoring performed at the last measured point of the study (40 days for embryos and 5 weeks for juveniles in the acute study, and 42 days for all life stages in the chronic study). All growth rates and adult feeding were analysed by repeated measures Analysis of Variance (ANOVA) while reproduction was analysed using a Kruskal-Wallis test due to the data not being normally distributed. Adult lipid, combined protein and CHO content, and movement were analysed using Analysis of Covariance (ANCOVA) with dry weight and shell length used as covariates respectively.

4.4. Results

4.4.5. Nanoparticle characterisation

Particle size

Mean z-average data for sonicated and settled 1mgL^{-1} CB suspensions at high and low calcium are displayed in Figure 4.2.

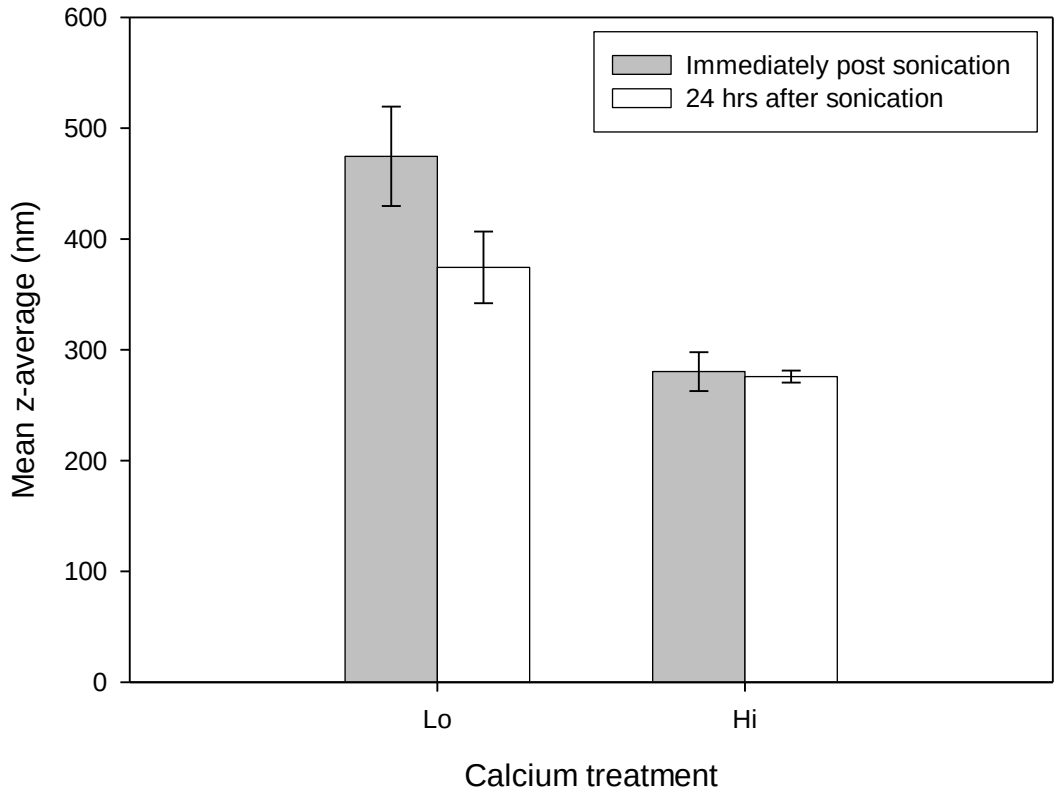

Figure 4.2. Mean size of CB at high and low calcium treatments. Data derived from a single sample measured in triplicate. Error bars denote standard deviation from these three readings.

Size distribution of particles was found to be unimodal in both the high and low calcium media. The diameter of particles was greatest in the 40mgL^{-1} (low) calcium medium and that both the high and low calcium media displayed an average diameter above 200nm (Figure 4.2). The 40mgL^{-1} calcium suspension appears to display smaller mean particle size after settling which is likely to be due to the fact that only the supernatant was sampled and larger agglomerates are likely to have settled during the elapsed 24hr since sonication.

Zeta potential

Figure 4.3 displays the mean zeta potential measured immediately after sonication and 24hrs after allowing for settlement, in high and low calcium media. The high calcium suspensions had the highest absolute zeta potential value and the smallest aggregates/agglomerates, which suggest that particles (or aggregates/agglomerates) are likely to stay in suspension longer in this treatment. In both calcium treatments, as expected, zeta potential decreased, as did the aggregates/agglomerates sizes, over the 24 hr period, which reflects the settlement of particulates that took place over that time interval.

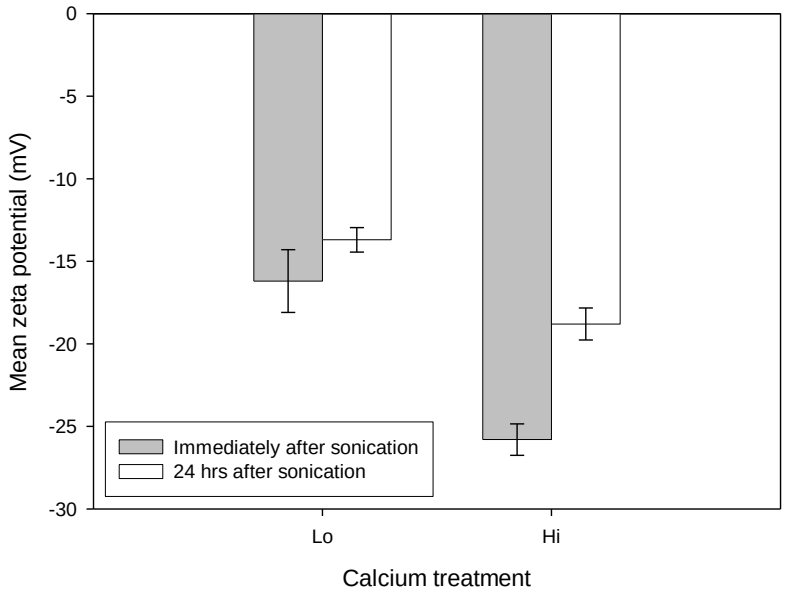

Figure 4.3. Mean zeta potential of CB at high and low calcium treatments. Derived from a single sample measured in triplicate. Error bars denote standard deviation.

4.4.6. Acute exposure

4.4.6.1. Embryo exposure

No significant effect of carbon black on embryo survival was observed throughout the course of the study (Wilcoxon log rank test, $X^2 = 2.54$, df = 3, p = 0.465, see Fig 4.4).

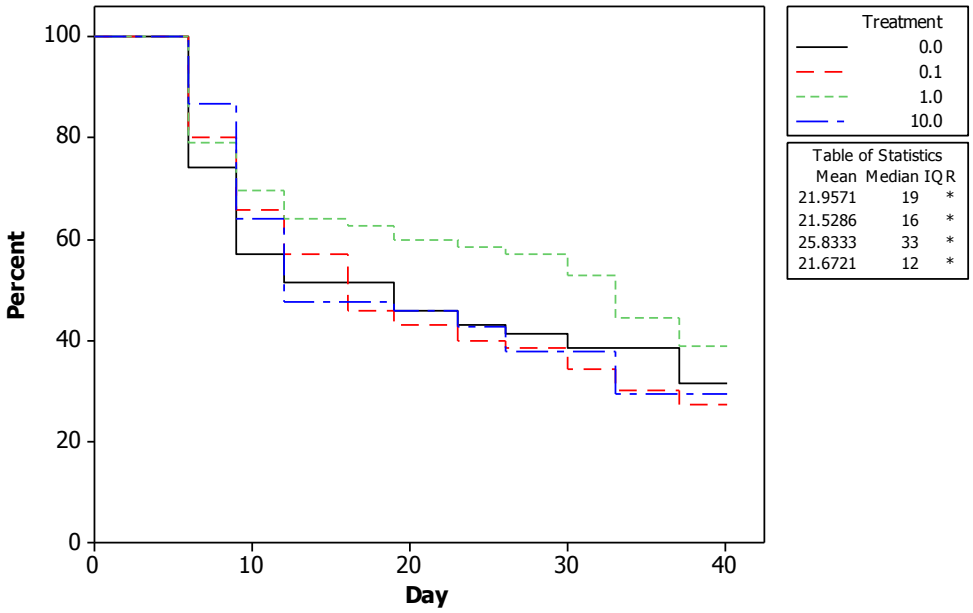

Figure 4.4. Nonparametric survival plot (Kaplan-Meier analysis) of time vs. treatment for embryos. Right censored at day 40 of exposure.

The highest concentration of CB displayed significantly lower embryo growth relative to the other groups. Figure 4.5 displays the mean length of surviving embryos after 40 days exposure and it can be seen that the $10mgL^{-1}$ group are significantly smaller than both the control and $1.0mgL^{-1}$ group but not the $0.1mgL^{-1}$ group (ANOVA, F = 6.24, df = 3, P = 0.001). It is worth noting, however, that the $10mgL^{-1}$ group took longer under the microscope to determine viability due to the CB particles coating them, thus increased stress caused by handling and the microscope light may have contributed to this result.

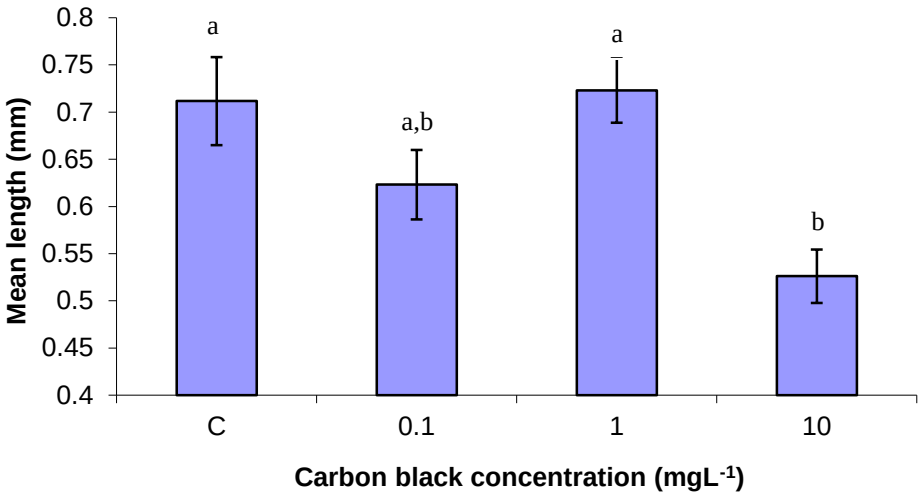

Figure 4.5. Mean length of embryos after 40 days exposure. Error bars denote standard error of mean, Bars with same letter are not significantly different (Tukey post-hoc test, p > 0.05).

4.4.6.2. Juvenile exposure

There was a significant effect of CB on juvenile survivorship, with highest mortality being found in the highest concentration groups (Wilcoxon log rank test, X^2 = 12.0, df = 4, P = 0.017, see Figure 4.6).

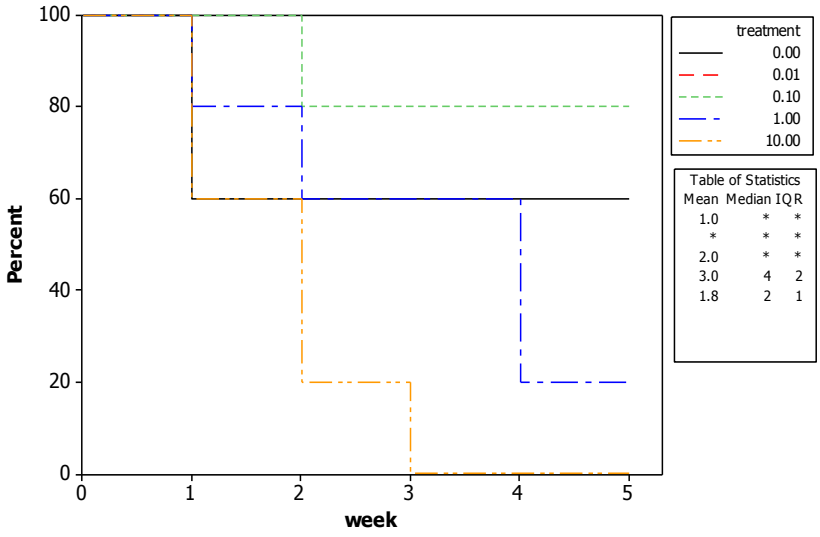

Figure 4.6. Nonparametric survival plot (Kaplan-Meier analysis) of time vs. treatment for juveniles. Right censored at week 5 of exposure.

Two deaths occurred in the control group in the first week of the exposure (most likely due to crushing during handling) and due to the small number of replicates (5) only 60% of the controls remained alive at the end of the procedure.

Juvenile growth, defined as total length increase over time (mm/week), was found to be significantly lower in the 1mgL^{-1} group relative to the 0.1mgL^{-1} group and controls (repeated measures ANOVA, F=14.567, df = 3, 10, p=0.021, see Figure 4.7). The 10mgL^{-1} group displayed 100% mortality by week 3 (see Figure 4.6) thus growth rates could not be calculated.

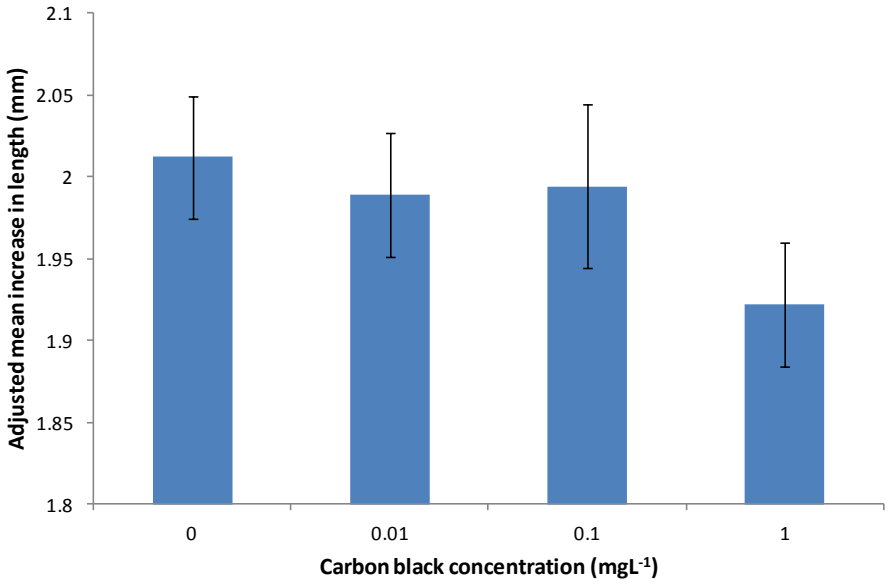

Figure 4.7. Adjusted mean increase in length of juveniles (mm/week) in different carbon black treatments. Error bars represent standard error of mean.

4.4.6.3. Adult exposure

Adults were found to feed significantly more in the 1 and 100mgL^{-1} groups (repeated measures ANOVA, F = 3.34, df = 3, 31, P = 0.032, see Figure 4.8). There was also significant variation in the amount of food consumed in different weeks, with all treatments showing a consistent drop in food consumption in week 2, which increased again in week 3. No interaction was found between terms. The adult shells were shown to be prone to chipping

when measured with vernier callipers and adult growth measurements are considered to be inconsistent and are not presented here.

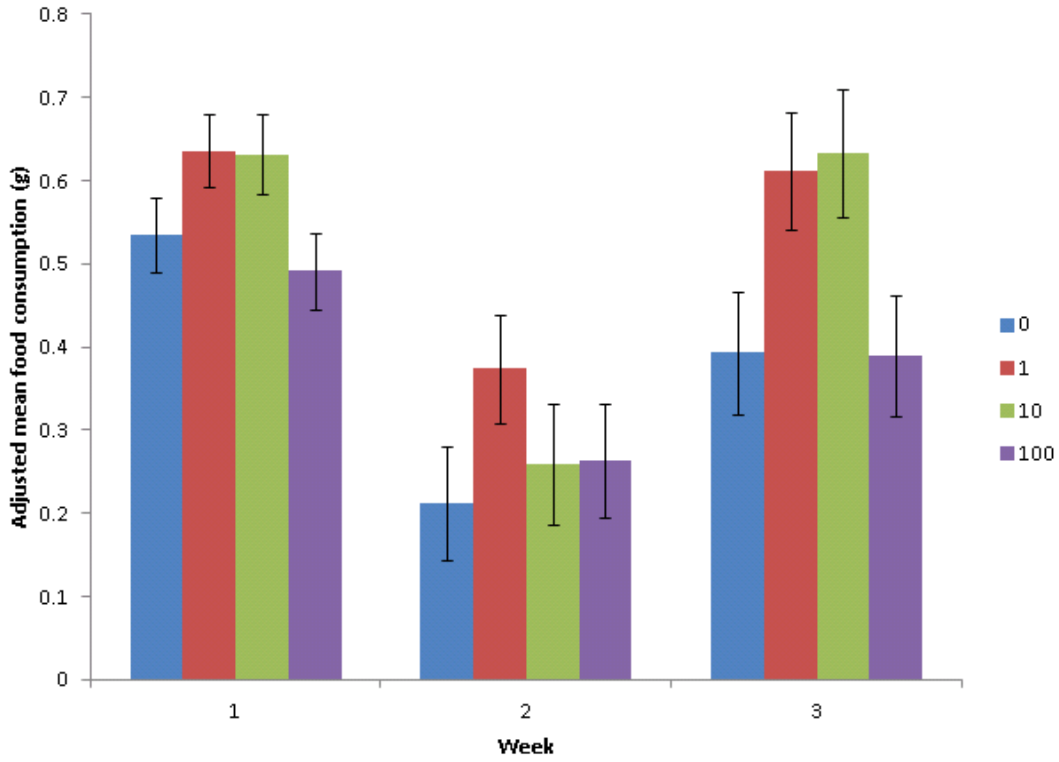

Figure 4.8. Adjusted mean mass of food consumed in different weeks of the exposure, for treatment and control groups. Legend items represent dose of carbon black in mgL^{-1}. Error bars represent standard error of the mean.

Higher reproductive output was observed in the highest CB concentrations. Significant increases in both the number of egg masses (Kruskal-Wallis test, H = 11.79, df = 3, P = 0.036) and the total no. of eggs (Kruskal-Wallis test, H = 12.20, df = 3, P = 0.015) produced were found in the higher CB concentrations (See Figures 4.9 and 4.10). The number of eggs per mass was not found to differ significantly across treatments (Kruskal-Wallis test, $P >$ 0.05).

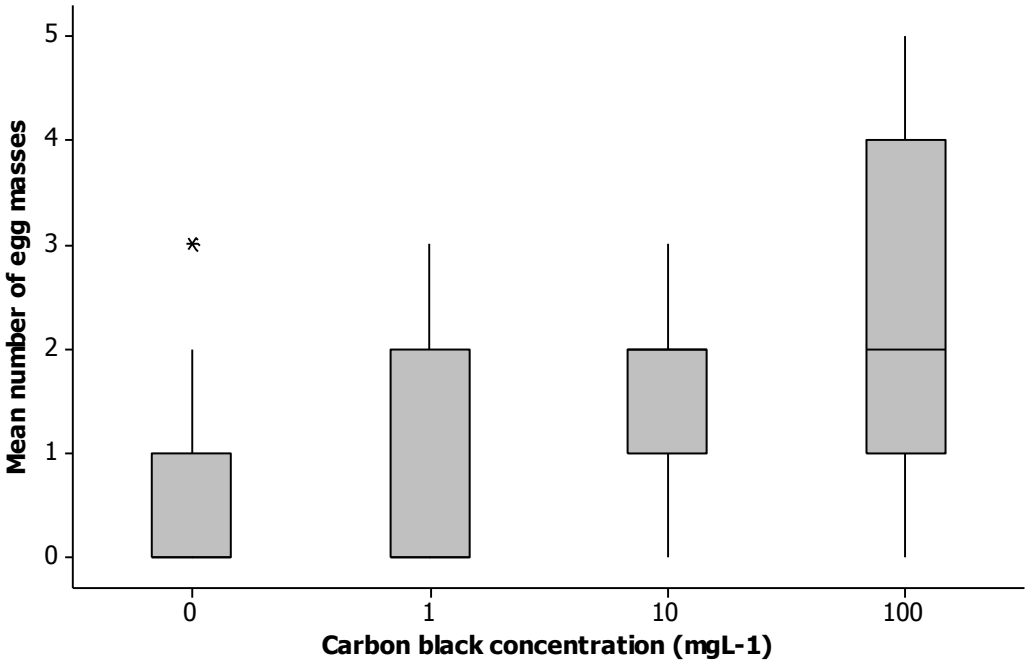

Figure 4.9. Boxplot of egg masses produced per individual throughout the study.

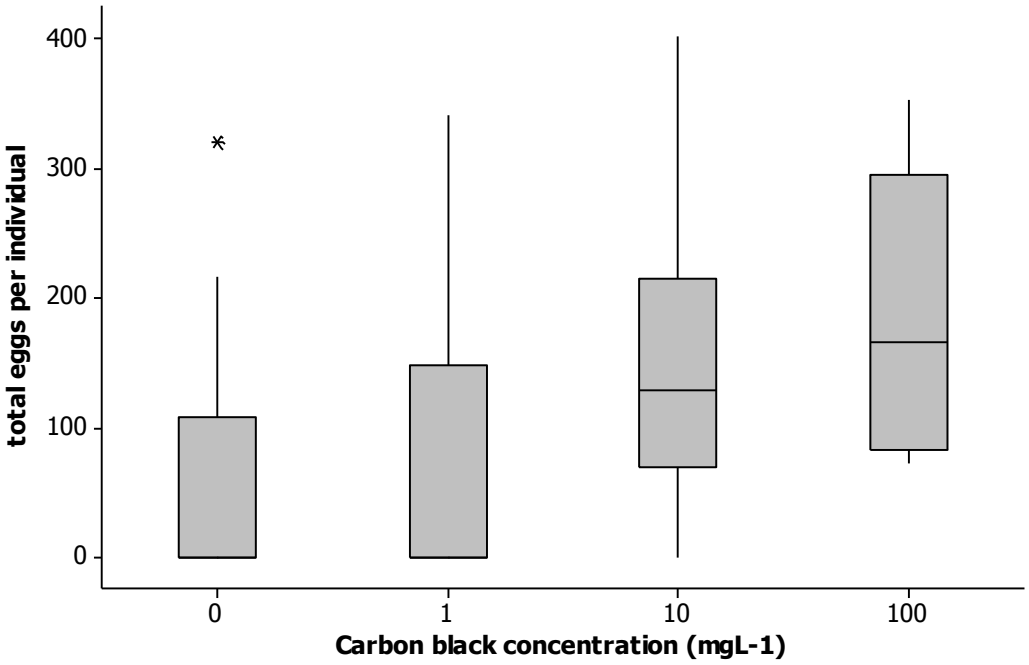

Figure 4.10. Boxplot of number of eggs per individual produced throughout the study.

4.4.6.4. Movement analysis

The results of the movement analysis are displayed below in Figure 4.11. Initial analysis indicated that length did not significantly affect displacement when added as a covariate (ANCOVA, P = 0.591, F = 0.295, df = 1, 31). Therefore a standard one way ANOVA was used to test differences between treatments. The results indicate that the control and the 100mgL^{-1} differed significantly in their displacement after 30 seconds from the controls (ANOVA, F = 4.72, df = 3, 31, P = 0.008). The 100mgL^{-1} group showed approximately half the displacement of the control group after the same amount of time.

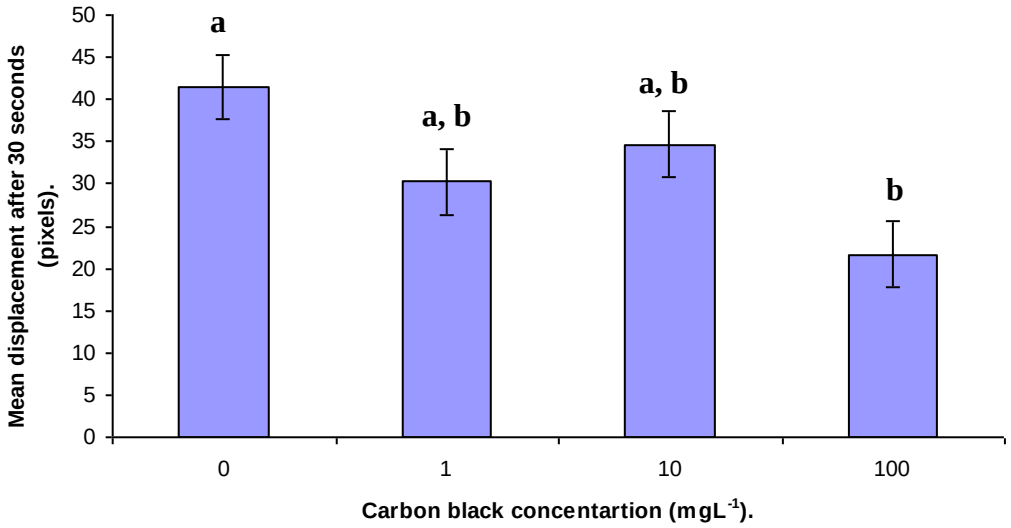

Figure 4.11. Mean displacement of different treatment groups after 30 seconds. Same letters denote means with no significant difference (Tukey test, P>0.05). Error bars denote standard error of mean.

4.4.6.5. Fat, protein and carbohydrate content

The results of the fat extraction undertaken on adults are displayed in Figure 4.12. The control group were found to have significantly more fat reserves relative to dry weight than the 100mgL^{-1} group (ANOVA, P = 0.039, F = 3.16, df = 3, 30). Total protein and carbohydrate content did not differ between treatments (ANOVA, P = 0.330).

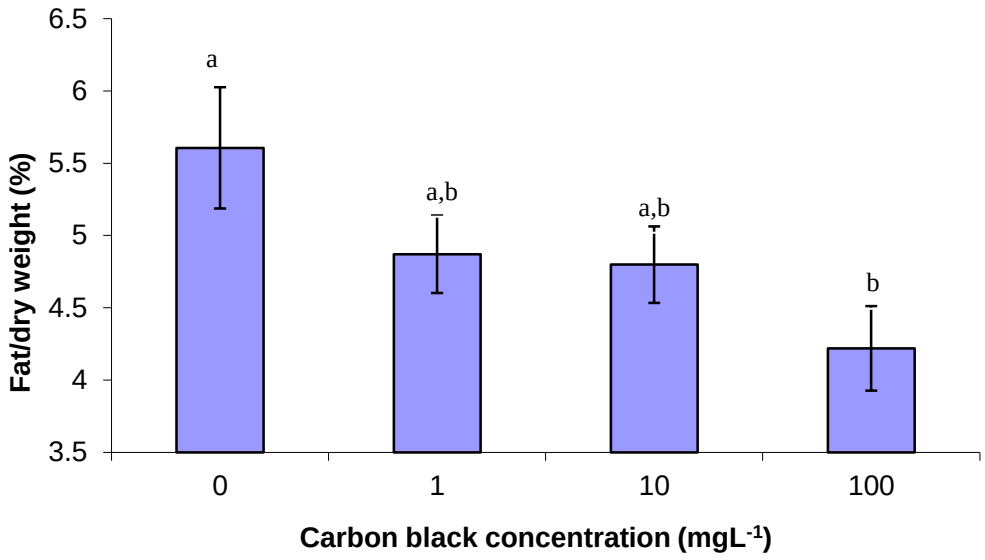

Figure 4.12. Mean percentage fat content after 3 weeks chronic exposure. Same letters indicate no significant difference between groups (Tukey test, P > 0.05). Error bars denote standard error of mean.

4.4.7. Intra specific variation in chronic CB exposure effects under different environmental conditions

4.4.7.1. Hatchlings

The survivorship plot for all populations is displayed in Figure 4.13. A significant difference in hatchling survivorship was found between populations (Wilcoxon log-rank test, χ^2=45.10, df=5, *P*<0.001). Pairwise comparisons revealed that both the Savernake (Wilcoxon test, χ^2=20.67, df=1, *P*<0.001), and Fauldhouse (Wilcoxon test, χ^2=5.67, DF=1, P=0.017) populations displayed significantly higher mortality in the low calcium CB exposure groups when compared to the high calcium groups (see Figures 4.14 and 4.15).

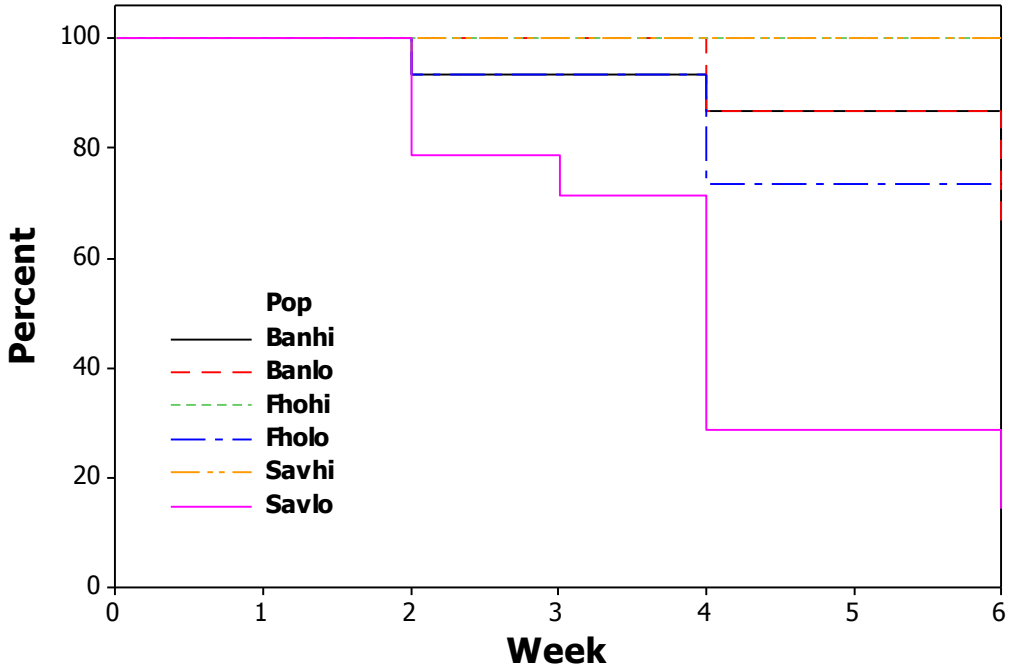

Figure 4.13. Nonparametric survival plot (Kaplan-Meier analysis) of time vs. treatment for hatchlings – all populations and calcium treatments. Right censored at week 6 of exposure.

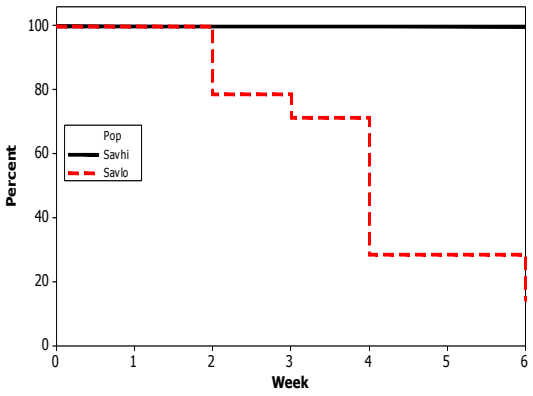

Figure 4.14. Savernake survivorship plot

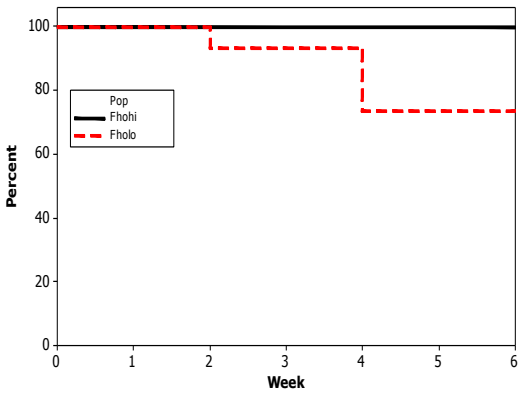

Figure 4.15. Fauldhouse survivorship plot

Hatchlings from the Savernake population were found to be particularly sensitive to low calcium with more than 80% mortality occurring in the low calcium treatment by the end of the 42 day exposure (Figure 4.14) compared to 25% mortality in the same treatment in the Fauldhouse population (Figure 4.15). No significant difference in mortality between calcium treatments was found for the Bank Well population (*P*=0.522).

Hatchling growth

Repeated measures ANOVA (incorporating initial size as a covariate, F=43.48, df=1, 55, P<0.001) revealed a significant difference between populations (F=12.30, df=2, 55, P<0.001) and an interaction between calcium treatment and population (F=7.48, df=2, 55, P=0.001); calcium treatment alone had no significant effect on hatchling growth (P=0.663) (see Figure 4.16). Growth differences between populations are only apparent in low calcium media. The interaction between calcium treatment is apparent as the low calcium Fauldhouse population was found to grow larger (both in relation to the other high calcium treatments and all low calcium populations) while the low calcium Bank and Savernake populations display reduced growth both in relation to the equivalent site high calcium populations and to the low calcium Fauldhouse population.

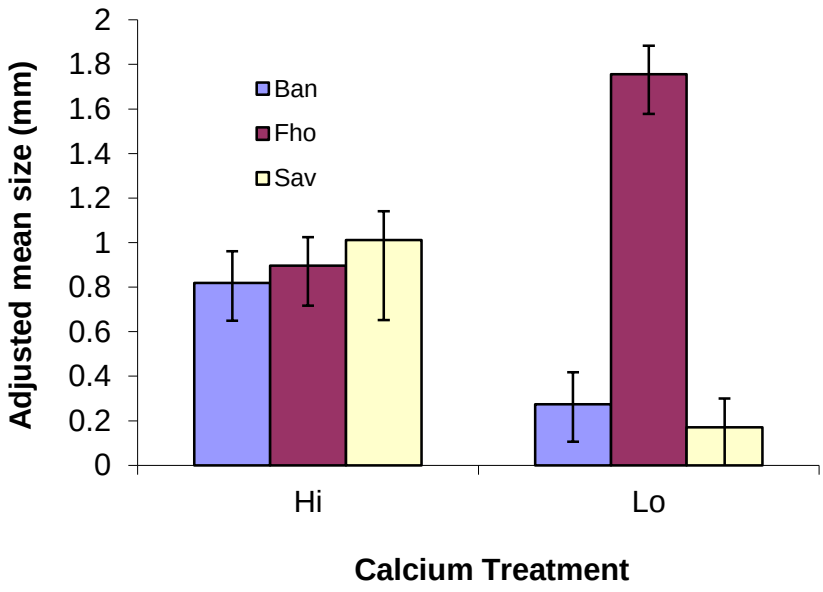

Figure 4.16. Adjusted mean size of hatchlings from different populations under high and low calcium treatments. Error bars denote standard error of mean.

4.4.7.2. Juveniles

Juvenile survivorship and growth

No significant difference was found between survivorship, with mortality only being recorded for one individual from the Savernake high calcium group in

the 6 weeks of the study. Repeated measures ANOVA with initial size as a covariate (F=349.99, df=1, 76, P<0.001) showed significant differences in growth between populations (F=5.73, df=2, 76, P=0.005) and calcium treatments (F=7.91, df=2, 76, P=0.006), but the interaction was not significant (P=0.078). Pairwise comparisons revealed that the low calcium treatments for the Savernake and Fauldhouse populations grew significantly larger while the Bank population appears to remain unchanged. The Fauldhouse population grew significantly larger relative to the other populations across both calcium treatments (Figure 4.17).

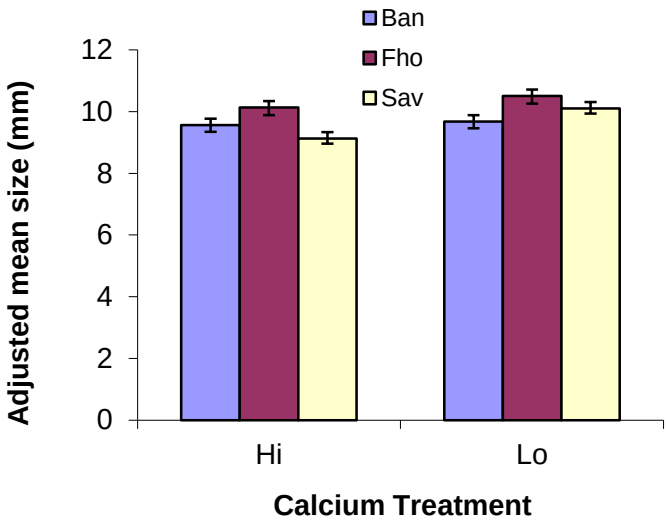

Figure 4.17. Adjusted mean size of juveniles from different populations under high and low calcium treatments. Error bars denote standard error of mean.

4.4.7.3. Reproductive adults

Adult survivorship, growth and reproduction

No significant difference was found in adult survivorship between population and calcium treatments (Wilcoxon test, χ^2=7.077, DF=5, P=0.215, Figure 4.18). No significant effects on growth were noted (repeated measures ANOVA, P <0.05). Only ten egg masses were laid across all the populations, throughout the study which was an insufficient number to allow statistical analyses to be performed.

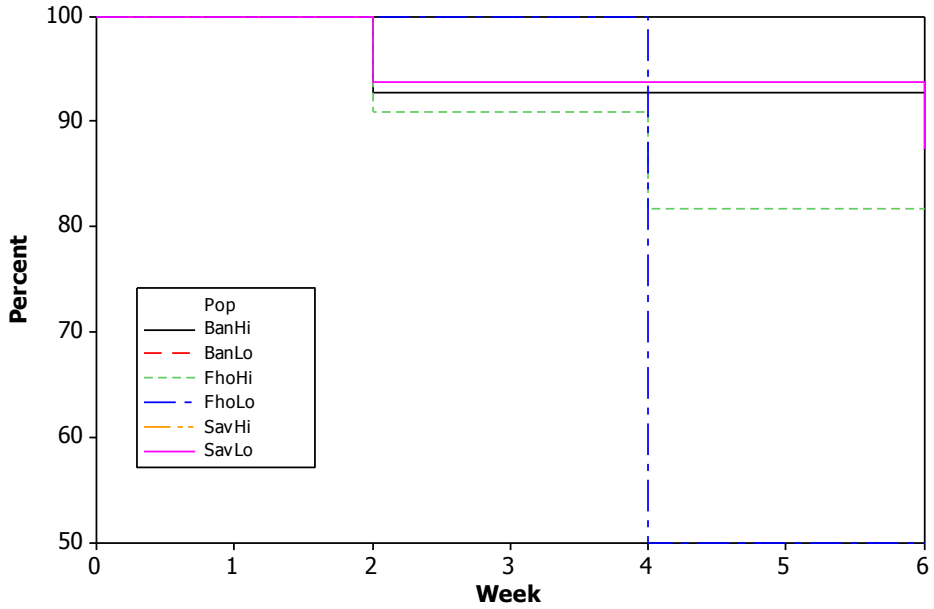

Figure 4.18. Nonparametric survival plot (Kaplan-Meier analysis) of time vs. treatment for adults – all populations and calcium treatments. Right censored at week 6 of exposure.

This was attributed to the fact that the low exposure volumes (30 cm^3) used to maintain constant mass dose in the different life stage exposures may have been stressful to the adults resulting in the lower reproductive output observed. Consequently, for matrix modelling (see Section 4.4.8) the data from F_2 reproduction (where individuals were exposed to varying calcium conditions alone) were used as part of the inputs (see Table 4.1).

Table 4.1. Reproductive data derived from F_2 generation and used as inputs to matrix model.

Population	Calcium	Hatching Time (weeks) ± SD	Mean no. Eggs/Week ± SD	Egg Survivorship (%) ± SD
Bank	Lo	3.77 ± 1.24	28.06 ± 10.2	0.80 ± 0.07
	Hi	3.70 ± 0.95	45.77 ± 9.44	0.74 ± 0.13
Fauldhouse	Lo	4.22 ± 1.46	72.67 ± 17.6	0.45 ± 0.19
	Hi	3.46 ± 0.94	50.58 ± 19.8	0.58 ± 0.27
Savernake	Lo	3.47 ± 0.59	19.56 ± 13.0	0.46 ± 0.26
	Hi	3.47 ± 0.78	30.63 ± 19.7	0.57 ± 0.14

4.4.8. Population level effects

The λ values and associated bootstrapped confidence intervals derived from the matrix model analysis are displayed in Figure 4.19. The Savernake population in the low calcium treatment had a significantly lower λ than all other treatments. Bank Well was also found to have a significantly lower λ than the Fauldhouse population in the low calcium treatment.

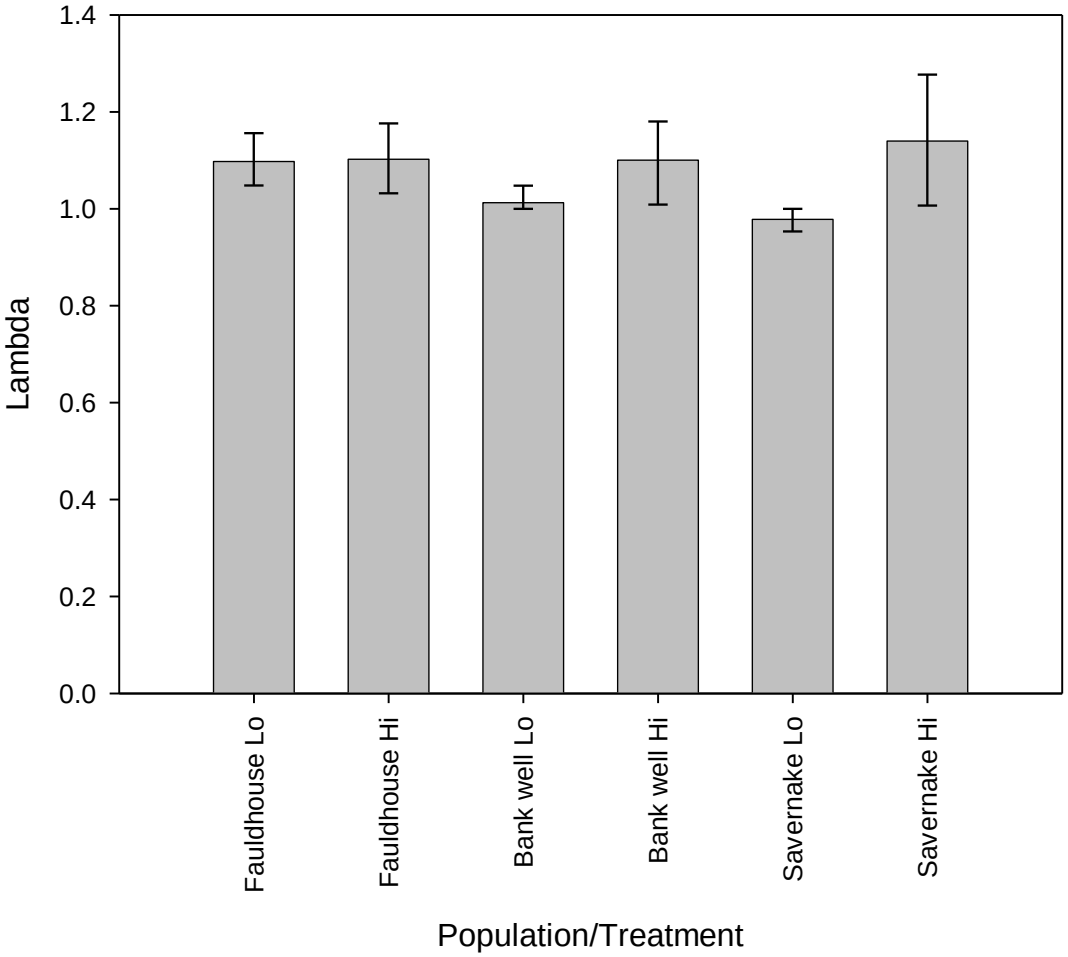

Figure 4.19. Lambda values for all populations at high and low calcium with bootstrapped confidence intervals.

In order to assess any differences in the influence of different matrix parameters on λ with varying calcium levels, the difference between elasticity values in the high and low calcium treatments was calculated (Figure 4.20). The difference in relative contribution of each parameter term to the overall λ value is represented by the size of the corresponding horizontal bars with positive values indicating an increase in the relative contribution of the parameter when moving from high to low calcium and a negative value

indicating a decrease in the contribution. For comparison, Figure 4.21 shows the equivalent data for the F_2 generation (see Chapter 3) of the same populations in high and low calcium treatments, but in the absence of nanoparticles.

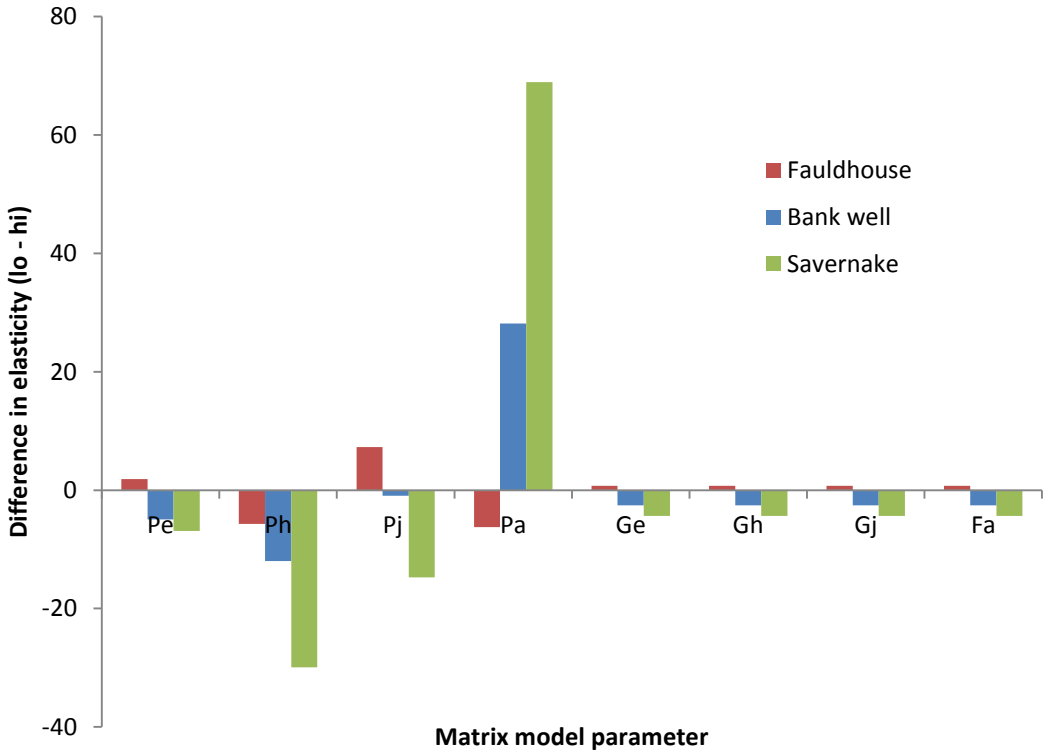

Figure 4.20. Difference in elasticity value between calcium treatments for each matrix parameter. Positive values indicate that the parameter has increased in importance in determining λ, and negative values that it has decreased in importance.

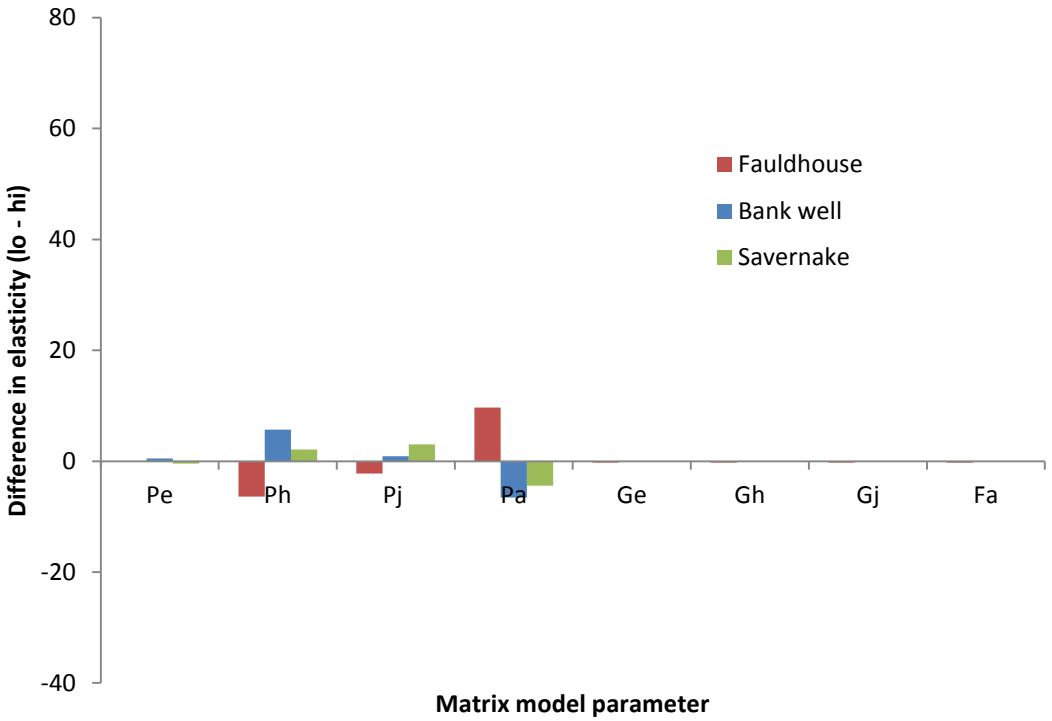

Figure 4.21. Difference in elasticity value between calcium treatments for each matrix parameter for the F_2 generation of the same populations in the absence of nanoparticles. Scaling of the *y* axis is the same as for Figure 6 for comparison. Positive values indicate that the parameter has increased in importance in determining λ, and negative values that it has decreased in importance.

4.5. Discussion

4.5.5. Particle characterisation

The particle characterisation analysis found that in both treatments the average diameter was above 200nm indicating substantial aggregation or agglomeration (Jiang et al., 2009). This is not surprising since it is expected that CB NP will aggregate/agglomerate when suspended in aquatic media. It is important to note, however, that DLS measurements tend to be biased towards larger aggregates/agglomerates and overall might not fully reflect the true size of aggregates/agglomerates present in these suspensions (Domingos et al., 2009).

For the low calcium treatment, there was a timepoint difference in mean particle diameter which was not apparent in the 200 mgL^{-1} calcium treatment.

Jiang *et* al (2009) reported for TiO_2 nanoparticles that as ionic strength in the media is increased there tends to be a decrease in zeta potential and a corresponding increase in aggregation. In Jiang et al's study pH was also shown to affect zeta potential and particle diameter, with an increase in particle size occurring as the isoelectric point (where the particles carry no net electrical charge) was approached and zeta potential becoming increasingly positive below, and increasingly negative above the isoelectric point. In the current study the fact that the lowest diameter particles were found in the highest ionic strength is most likely due to the fact that the increased calcium chloride salt concentrations are likely to bring about a slight increase in pH which may lead to smaller aggregate sizes (Jiang et al., 2009). The zeta potential values of the media tested were generally less than the | ± 30| mV limit which generally indicates a stable suspension in the absence of steric stabilisation (Jiang et al., 2009). This suggests that the suspensions were not particularly stable, which concurs with the observed high settlement over the period of measurement (24 hours).

4.5.6. Acute exposure studies:

Embryos

Carbon black exposure had no significant effect on embryo survival during the 5 weeks of exposure. However, mortality in all groups was approaching 60-70% after 5 weeks exposure (Figure 4.4). This is markedly higher than the level of embryo mortality observed in the studies reported in Chapter 2, and by Wagner (2000) where the survivorship of dissected egg masses was close to 100% in control groups after a similar time period. It is possible that the evident increase in mortality which appeared to result from the cutting of the egg masses may have masked any more subtle effects of exposure to CB, but it would appear that these effects are minimal based on current evidence.

In terms of growth (Figure 4.5), while the $10mgL^{-1}$ embryo group was found to be significantly smaller after the exposure period, it is possible that extended handling and observation under the light microscope, due to the carbon black preventing easy observation of the embryos, could also have been a factor in the observed results. Microscopic analysis revealed no evidence for

penetration of the eggs by CB. This does not rule out the possibility that CB was being transferred into the eggs. Embryonic stages are typically the most vulnerable to pollutants (Wagner, 2000) and the fact that mortality was not significant at the 10mgL^{-1} range over a 5 week exposure period would tend to indicate that CB exposure did not cause toxicity for the endpoint measured. Further studies could assess whether NPs are capable of penetrating the eggs of *L. stagnalis*. This could be tested by exposing the dissected mass of intact embryos to fluorescent nanoparticles, such as polystyrene beads, and then use confocal microscopy to visualise any fluorescence within the embryos or eggs themselves (Rosenkranz et al., 2009). Although of course CB NP will have different characteristics and size when compared with fluorescent polystyrene beads and so interaction and uptake may also differ.

Juveniles

Survivorship analysis in the juvenile snails (Figure 4.6) indicated a significant effect of treatment on juvenile survival with the 10mgL^{-1} group displaying 100% mortality by week three of exposure. The data appeared to follow a typical dose-response curve with the exception that 2 of the controls died by the first week of the study. Juvenile *L. stagnalis* are very fragile (Van Der Steen et al., 1969) and this mortality was most likely due to damage during handling (at least one of these individuals was shown to have a cracked shell under the microscope) as this was the first times the juveniles had been handled experimentally and the control group was the first to be handled. In the first week of exposure a plastic spoon was used to manipulate individuals which proved too damaging resulting in a wide-bore plastic pipette being used instead to move the juveniles via suction in subsequent weeks which caused less damage.

Growth was shown to be significantly affected by CB exposure (see Figure 4.7). Due to the fact that the highest exposure group had died by week 3 it was only possible to analyse the remaining treatment groups and controls by repeated measures ANOVA. Lowest growth was observed in the 1.0mgL^{-1} group and the remaining groups appeared to follow a dose-response

relationship indicating that CB exposure results in significant toxicity to juvenile *L. stagnalis*, leading to mortality and reduced growth rates.

The route of entry and any subsequent translocations of NPs within the snails are unclear at this point but it is surmised that the majority of particles were ingested as the faeces of exposed individuals were black indicating that CB particles had been passed through the digestive tract. From there it is possible that NPs may be translocated to other parts of the body, although the majority may pass through the body without being absorbed (Roberts et al., 2008, Rosenkranz et al., 2009). Another likely route of entry is respiration as surface breathing may take in particles caught in the surface film of the water. Further analysis of specific tissues using fluorescent particles and confocal microscopy would allow these routes of entry to be confirmed and any possible organs of accumulation of NPs to be identified.

Adults

Of the 40 adult snails involved in the adult snail study only four displayed mortality (one in the controls and the 100mgL^{-1} group and two in the 10mgL^{-1} group). This is in keeping with previous studies that suggest that the adult life cycle stages in *L. stagnalis* are less sensitive to the impacts of pollution (Coeurdassier et al., 2003, Desouky, 2006, Elangovan et al., 1997, Wagner, 2000).

Feeding

Adult snails were shown to feed significantly more in the 1 and 10mgL^{-1} groups relative to the control group, while the highest concentration group (100mgL^{-1}) showed no significant difference from control feeding (see Figure 4.8). This contrasts slightly with previous studies of similar species such as *Physa acuta* which showed reduction in feeding with increased concentrations of ionic liquids (Bernot et al., 2005). The increased feeding observed in the lowest concentration groups may be associated with the increasing energetic costs of detoxification where more food is required to maintain physiological functions. Another possible cause of the observed effects could be due to the

mechanical blocking of the digestive tract by CB particles causing less nutrients to be absorbed (Oberdorster et al., 2005).

Reproduction

Exposure to CB caused increased reproduction to take place in the higher exposure groups (see Figures 4.9 and 4.10). Reproduction was shown to increase both in terms of the number of egg masses (Figure 4.9) and number of eggs per individual (Figure 4.9) throughout the course of the study. The snails in the 10 and 100 mgL-1 groups displayed levels of reproduction three times higher than the controls, suggesting a marked up regulation in direct reproduction (termed fecundity compensation) in the face of a perceived immediate threat to survival (Zbikowska et al., 2006). Fecundity compensation also occurs in *L. stagnalis* when it becomes infected by trematode parasites (Ballabeni, 1995). At the point of perceived infection *L. stagnalis* reproduction dramatically increases before parasitic castration sets in and the phenomenon of gigantism may occur as snails divert energy freed from reproduction solely into growth (Zbikowska et al., 2006). Ballabeni (1995) suggests that such a response may serve to increase ultimate fitness in the face of a temporal threat (parasites) and that by investing only in growth *L. stagnalis* increases the probability of outliving the trematode infection. Again the results obtained here contrast to some extent with other toxicological studies, which typically show reduced reproductive output in response to toxins (Coeurdassier et al., 2003). It is possible that exposure to NPs engenders a different response from other toxicants, although if that is the case the reasons why this might be remain unclear. Certainly, the results observed in this study more closely resemble those seen in response to parasite infection as discussed above, or to endocrine modulating substances which act directly on hormonal signalling involved in reproduction (Czech et al., 2001).

Fat content and movement

The results of the fat extraction indicate that the 100mgL^{-1} group contained significantly less fat reserves than the control group (see Figure 4.12). Fat reserves are a very important form of energy storage in snails (Duncan et al.,

1987) and a reduction in fat reserves will be representative of a decreased state of physiological health, rendering individuals less likely to survive (Rigby and Jokela, 2000). The results of the fat extraction displayed in Figure 4.12 indicate that individuals in the highest treatment groups were using fat reserves as a result of exposure to CB.

No significant differences in combined protein and carbohydrate content were noted across treatments indicating that response to CB affected fat reserves before other long term types of energy storage.

Adult *L. stagnalis* treated with CB displayed significantly less displacement in the 100mgL^{-1} treatment group than the control group. The results displayed in Figure 4.11 indicate that CB caused reduced movement in all treatment groups, although this was significant in only the 100mgL^{-1}. The causal nature of these observations is unclear; it is possible that CB interacts with *L. stagnalis'* ability to produce mucus, acting as an abrasive and reducing the snail's ability to move normally. It is of interest to note that the results of the movement analysis mirror the results of the fat extraction (see Figure 4.12). The symmetry of the movement and fat data suggests that movement/displacement analyses may serve as a non-invasive means of assessing physiological stress *in vivo* and may be of further use in the life history analyses at a later date. Further analysis of the data involving directedness and displacement may reveal whether the behaviour of the snails has been altered by CB exposure and may indicate that neurological damage has occurred as a result of exposure (Salanki et al., 2003).

Summary of Observed Effects: Energy Partitioning.

In adult *L. stagnalis* exposure to CB resulted in increased feeding at low concentrations while the highest CB concentration resulted in reduced feeding relative to controls.

This response could be due to the increased energetic demands of detoxification, whereby a 'threshhold' level between detoxification and toxic response is exceeded in the highest CB concentration. Increased

reproduction was also found to occur in the highest exposure groups, perhaps representing a compensatory response toward a potentially lethal substance. Members of the family Lymnaeidae are known to increase reproductive output in the initial phase of parasitic infections (Ballabeni, 1995) and it is possible that the observed reproductive effects are an adaptive trait to increase ultimate fitness in the face of temporal biotic or abiotic threats. The results of the feeding and reproductive output studies were mirrored in the fat reserve study, whereby the lowest relative fat content was found in the highest exposure group, which had also invested heavily in reproduction throughout the study. The schematic summary displayed in Figure 4.22 reflects the partitioning of the energy budget between maintenance, growth and reproduction in the face of increased cost of detoxification.

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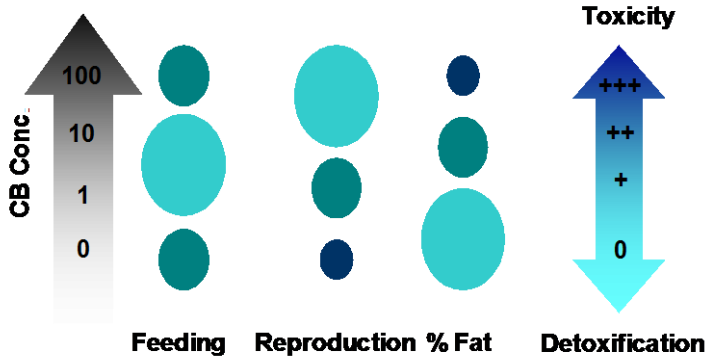

Figure 4.22. Schematic representation of the observed effects of CB particles on *L. stagnalis*. Size of 'response' (feeding reproduction and % fat content) is indicated by the size of the circles

4.5.7. Intra specific variation in chronic CB exposure effects under different environmental conditions

The exposure of different life cycle stages of *L. stagnalis* to CB NPs in this study clearly identifies that different stages vary in their sensitivity. The greatest effects, both in terms of mortality and growth, were observed in the hatchling group whereas adult animals display the least response to CB exposure, although the detection of effects on adult fecundity was limited by the lack of data due to experimental conditions (limited cup volumes). From the results, it is also evident that the effect of CB NP exposure is influenced

by other environmental stresses (in this case calcium availability), but that responses to the interaction between the two vary between populations. For example the hatchling stage displayed little mortality when reared in high calcium media, with no mortality occurring in the Savernake and Fauldhouse populations, and only 20% occurring in the Bank Well population. In contrast higher mortality was found in all the low calcium exposure groups, with both Savernake and Fauldhouse (Figure 4.13) differing significantly from their high calcium counterparts.

Between population effects were also observed in growth of both the hatchling and juvenile groups (Figures 4.16 and 4.17). In the instance of the hatchlings the effects of CB exposure were not conserved across calcium treatments (i.e. there was a significant interaction) with the Bank Well and Savernake populations both displaying significantly lower size (and therefore growth) relative to their high calcium counterparts, while the low calcium Fauldhouse group displayed a significantly larger size.

In the juvenile stage, there was a significant effect of calcium availability on growth, but in this case growth appeared to be enhanced at low calcium levels. Although there was no significant interaction of calcium with population, the increase in growth was most pronounced for Fauldhouse, and secondarily Savernake populations. It is possible that the increase in growth was a compensatory mechanism to offset effects of lower survival at the hatchling stage by speeding the development towards the reproductive adult stage. This is further examined in the population matrix models presented later in this chapter. Alternatively, due to the evident differences in sensitivity of different life stages to CB and environmental stress effects, it is possible that the combination of experimental conditions used corresponded to levels of stress which would elicit a different extent of response (life-table response experiments), (van der Ploeg et al., 2011). For the hatchlings, their enhanced sensitivity could have led to responses reflecting direct toxicity effects. However for less sensitive juveniles, the low calcium and CB treatment could have elicited a hormetic response, as has been found in other studies (Lefcort et al., 2008) resulting in enhancement of growth.

Population and calcium effects were also found in the juvenile exposure groups (Figure 4.17) but in this instance calcium displayed no interaction across populations. It would appear that all populations tended towards increased growth in the low calcium media at this life stage.

Population-level differences in the response to CB exposure under different levels of calcium availability could result from differences in genetic history, underlying levels of genetic variation within populations and the extent of local adaptation of prevailing environmental conditions (Ducrot et al., 2010, Lopes et al., 2006, Pease et al., 2010, van Ooik and Rantala, 2010). When exposed to similar conditions, these factors influence both the extent to which individuals can respond to stresses (Duchet et al., 2010), here in the form of CB nanoparticles, and the mechanisms or life history traits which show responses, resulting in differential patterns of response across the populations when exposed to the novel anthropogenic stressor.

Although it is not possible to generalise effects across the different life stages and endpoints, it could be argued that they were more acute in low calcium exposures. Particle characterisation data indicated that aggregates in high calcium exposures tend to be smaller and potentially more stable in suspension. It could therefore be argued that exposures to the nanoparticles would be higher in the low calcium medium, given the propensity for the larger aggregates to settle and therefore being available to the snails. In the context of this experiment, however, it is likely that settlement of most aggregates would take place over the weekly period, prior to media replacement, and therefore any differences in this context between media are likely to be negligible.

4.5.8. Population level effects

The use of matrix models allows the response of the different life stages to be combined and assessed at the population level via calculation of the

population growth rate, λ. None of the populations in this study reared in the high calcium media were found to have a value of λ below 1 (Figure 4.19) and as such can all be considered to be showing positive population growth despite the burden of CB nanoparticle stress. However this study does not take into account other environmental stressors such as predation and the lambda values reported here are likely to be reduced further in the field due to these effects (Sandrine et al., 2009). Again due to the use of data from non-CB exposed adults for the fecundity parameter it is possible that the effects on adult stages have been underestimated to some extent.

There were clear population differences in the extent to which calcium availability influenced the population level response to CB exposure. The Fauldhouse population was seemingly unaffected by differences in calcium availability in terms of population growth rate. The changes in survival evident in the low calcium treatment may be compensated for in population terms through a trade-off with increased subsequent growth, as was evident in the experimental results. Such trade-offs are evident in life-history responses to a wide variety of stressors and other selective forces (Dudycha and Tessier, 1999, Lewis, 2001). The Fauldhouse population was introduced, and as such may show adaptation of life history through local genetic differentiation in a way which has allowed it to be relatively insensitive to calcium concentrations, despite reduced survival of hatchlings compared to other populations.

In contrast, both Bank Well and Savernake showed a reduction in λ in low calcium treatments, with the Savernake population differing significantly from all the other populations with a λ below 1, indicating that this population would eventually become extinct. It would therefore appear that populations differ in their responses to CB nanoparticle stress, but that the degree of response is constrained by the environmental context (in the form of calcium availability) that the populations are reared in.

The results of the elasticity analysis displayed in Figure 4.20 indicate the difference in relative contribution of each model parameter towards the overall λ value between high and low calcium treatments. Consistent with the

insensitivity of the population growth rate to calcium treatment, it can be seen that the Fauldhouse population displays little difference in elasticity between the high and low calcium treatment. Hatchling and adult survivorship (terms Ph and Pa) both show a small negative difference indicating that these parameters contributed slightly more to λ in the high calcium population while juvenile survivorship (Pj) contributes more in the low calcium population, consistent with the experimental results. By contrast both the Savernake and Bank Well populations display a broadly similar response in terms of changes in parameter contributions across calcium treatment, but to different degrees. The greatest difference in contribution to λ was found in the adult survivorship parameter (Pa), with the Savernake population displaying the largest shift between calcium treatments. Like the Fauldhouse population, hatchling survivorship (Ph) was also found to contribute more towards λ in the high calcium groups (resulting in negative differences) with again the greatest difference being found in the Savernake population. By considering the observed differences in elasticity it would appear that the larger adult survivorship (Pa) contribution to λ in the low calcium groups for the Bank Well and Savernake populations is complemented by a reduction in the contribution from hatchling survivorship (Ph). In the case of the low calcium Savernake population, the contribution to λ from the Pa parameter has become so dominant that this population could be characterised as one dominated by adults with little survival past the hatchling stage taking place. The same pattern to a lesser extent is mirrored in the Bank Well low calcium population resulting in a reduced λ value of 1.01 (95% CI lower, 1.00, upper, 1.05) indicating that the Bank Well population will tend towards a stable growth state and that the contribution to population growth rate will be derived more from the adult age class and less from the hatchling stage, which conversely is most impacted by compounding stressors. In comparison with the changes in elasticity shown between calcium treatments by the different parameters in the absence of nanoparticles (Figure 4.21) there is clearly a substantial increase in the magnitude of changes in relative contribution of different parameters in different treatments. In addition the pattern of variation also differs. Pa and Ph are still the traits that show the most substantial changes, but the variation between high and low calcium is commonly

reversed when comparing the results with and without nanoparticles. This would tend to suggest that the life history response shown in relation to nanoparticle exposure and differential calcium availability is quantitatively different to that shown to variation in calcium availability in the absence of other stressors.

There is general agreement between the life history traits which show the greatest sensitivity to changes in calcium concentration and those which were identified as showing most significant change in elasticity in relation to change in calcium (i.e. those with a negative change in elasticity). This runs counter to a recent review (Forbes et al., 2010) which found that traits with high sensitivity to toxicants had generally lower elasticity, although this conclusion is acknowledged to be somewhat tentative given the range of model organisms included in the review was limited and confounding factors could not be excluded. Other studies using mollusc species (Salice and Miller, 2003) have come to similar conclusions as the present study in relation to trait sensitivity and contribution to population growth rate, so this remains an area where more work is needed to establish if any generality is possible.

4.6. Conclusion

Acute exposure to CB appears to affect feeding, locomotion, reproduction and body condition in *L. stagnalis*. Juveniles are more vulnerable than adults and display significant mortality in acute exposures while little effect was observed on embryos, perhaps due to the inability of CB particles to penetrate the eggs. The observed responses are consistent with potential partitioning of energy between different physiological functions (e.g. growth, reproduction and detoxification).

There are not many published studies where the effects of CB NPs have been assessed on taxa other than human models. Studies using algae (Nielsen et al., 2008) and mussels (Canesi et al., 2008, Canesi et al., 2010) were undertaken in the marine environment, where NPs will behave differently. Nevertheless, there was indication from those studies that CB NPs may exert significant inflammatory effects in *Mytilus galloprovencialis* immunocytes,

when tested in vitro (Canesi et al., 2008) and may lead to changes in lysosomal and oxidative stress biomarkers in the digestive gland when tested in vivo in *M. galloprovencialis*, and that different life stages may show different sensitivity when exposed to CB NP (Nielsen et al., 2008).

Chronic exposure revealed intra-specific differences in response to low level CB exposure at different life stages. As with the acute study, early life stages were found to be more sensitive to CB with significant mortality only ocurring in the hatchling stage. Population differences in survivivorship and growth generally appeared more pronounced in the low calcium treatments suggesting that effects of anthropogenic CB NPs may be more detectable where enivronmental context implies a higher degree of stress (in the form of reduced calcium availability). The differential response across populations at detectable in both the chronic and population level studies indicates that the degree and magnitude of response to CB (and likely other anthropogenic stressors) is both population specific and dependant on the evironmental context of the receiving population.

Intra specific differences continued to be detected at the population level and were again dependant on environmental context in the form of calcium availability. Again, the results indicated significant intra specific differences in response to CB nanoparticle stress at the population level which only became apparent in the low calcium treatment, where environmental stress would be considered maximal. The magnitude of responses in terms of population growth rate was found to range from little detectable effect (in the Fauldhouse population) towards increasing severity in the Savernake population, where this population was found to tend towards extinction in low calcium environments when exposed to CB stress. The observed population differences are suggestive of local adaptation to prevailing environmental conditions (Lopes et al., 2006) and the adoption of different life history strategies, which can lead to substantially different responses to novel stressors.

In order to accurately model responses of organisms to potential toxicants at the population level it is necessary to take a whole life-cycle approach (van der Ploeg et al., 2011) to ensure that variable sensitivity of different life stages and the potential for trade-offs in life history traits are accounted for. Furthermore, the different population responses presented in this and other studies (Ducrot et al., 2010, Salice and Miller, 2003) highlight the need for toxicity testing to consider, where financially and practically possible, both population differences and the interaction between naturally occurring environmental stressors, in order to more accurately predict the actions of toxicants in wild populations.

This study therefore demonstrates the importance of considering more than one population of test organisms, as well as life stages, in conjunction with varying levels of environmental stress when attempting to conduct ecotoxicological testing that is representative of real life exposure.

5. General discussion

The aim of this study was to examine patterns of life history variation in different populations of *L. stagnalis* sampled from across the UK geographic range, in response to natural and anthropogenic stressors. This was achieved by addressing the main objectives described in section 1.3 which involved:

1) Analysing geographic variation in life history traits, and any trade-offs therein, in response to environmental calcium (Chapters 2 and 3);
2) Performing preliminary toxicological studies of the responses of *L.stagnalis* to nanoparticle CB at different life stages before subsequent selection of three populations to examine the combined effects of calcium and CB (Chapter 4);
3) Linking the life history data via a matrix model to examine the effects on population growth rate of calcium alone and combined with CB (Chapter 4).

This discussion chapter aims to link the main findings of the research carried out and evaluate the success in addressing the two main hypotheses defined in section 1.3.

The first hypothesis that was proposed was that life history adaptation to prevailing environmental conditions may involve different trade-offs across the geographic range due to genetic and phenotypic variation. In order to address this hypothesis it is first necessary to describe the nature and breadth of life history adaptation revealed in Chapter 2 of this study. This involves trying to pick out consistent patterns of variation between populations and across generations, and evaluating whether these are under genetic and/or phenotypic (environmental) control. Intra-population differences were consistently detected in most traits surveyed in Chapter 2 but in general these differences were not consistent across F_1 and F_2 generations indicating that a high degree of variation, derived from phenotypic plasticity, was inherent in most life history traits studied. Growth rates in molluscs are known to be sensitive to a number of environmental variables such as temperature, diet and water quality and chemistry (Boycott, 1936, Dillon, 2000, McMahon, 1983, Russel-Hunter, 1964). Traits pertaining to growth rates appeared to be

particularly plastic with none of the Gompertz growth model parameters displaying any correlation across generations. Similarly age at first reproduction was shown to be highly variable across generations and would suggest that this trait was sensitive to environmentally derived differences in growth. Differences in size at first reproduction were detected between populations and, despite displaying plasticity across calcium treatments, a significant correlation between the F_1 and F_2 generation indicated that these differences were conserved and likely to be under genetic control, suggesting that local adaptation in this trait had occurred across the sample populations. Similar trends were observed in reproductive traits although a greater degree in plasticity in some aspects of these traits is discussed later. Studies by Lam and Calow (1989a, 1989b) and Brown (1983) found that differences in life history in freshwater gastropods tended to be characterised by variation in the same traits described here, which were shown to be under genetic and environmental control (Dillon, 2000). These findings are in keeping with the observations of Price and Schluter (1991) who state that life history traits directly tied to fitness tend to be subject to more efficient natural selection and thus display lower heritabilities than other traits (such as physiological or behavioural traits) with less direct links to fitness.

In order to fully address the hypothesis above it is necessary to consider the relationships between traits as were examined in Chapter 4. Evidence in support of locally adapted variation via trade-offs in life history traits comes from the observed relationship between size and age at first reproduction and reproductive output. Here reproductive output was shown to be related to adult size, while both these characteristics were shown to trade-off against age at first reproduction. Such trade-offs between growth and reproduction are common in animals with indeterminate growth (Heino and Kaitala, 1999, Stearns, 1989b) and are likely to be influenced by age specific mortality, particularly at early and post reproductive life stages (Stearns, 1992), which was not analysed in this study. Age at first reproduction appeared to be more responsive to environmental influence than size at first reproduction, which displayed less variation. This suggests, unsurprisingly given the findings of this study and that reproductive output is typically a factor of adult size in

freshwater molluscs (Dillon, 2000), that local selection has favoured a population specific size, rather than age, at first reproduction across the study populations. That size at first reproduction is correlated with reproductive output and that these traits are directly tied to fitness may explain the lower heritabilities found in these traits relative to the others surveyed (Price and Schluter, 1991, Stearns, 1983).

A further trade-off was detected between the number of egg masses and the number of eggs per mass. This relationship appeared to be stronger in the F_2 generation in both calcium treatments, while both traits (although eggs per mass was not significant) appeared to be conserved across generations suggesting that variation in these traits was under genetic control. Egg number was shown to be more strongly conserved across generations indicating that differences in reproductive strategies between populations were best defined by a negative relationship between the number of egg masses and the number of egg per mass. This trade-off could be reflective of local environmental stability, whereby more fluctuating environments could prompt more frequent reproductive events (egg masses) at the cost of having a reduced number of eggs per mass (Chapuis et al., 2007, Dillon, 2000). Variation in reproductive characters was also shown to be sensitive to environmental calcium, particularly in the number of eggs per mass which was shown to decrease in the low calcium treatments. The retention of such variable approaches to reproduction may also be reflective of the semelparous lineage of *L. stagnalis*. Semelparity is the norm for freshwater pulmonates and *L. stagnalis* has evolved an iteroparous reproductive strategy from this ancestral condition (McMahon, 1983), a move perhaps prompted by environmental instability (Dillon, 2000, Ranta et al., 2002).

Trade-offs were generally shown to involve the same traits across the study populations, namely those across reproductive traits and those relating to size and age at reproduction and reproductive output. Evidence to support population specific trade-offs (and the evolution of more distinctive life history strategies) is seen in the Epping and Fauldhouse populations. The Epping population was shown to have the highest juvenile mortality of all populations

and the highest reproductive output (both in number of eggs per mass and in total egg number) across both generations. Some further support for this trade-off between juvenile mortality and reproduction comes from the fact that Epping population produced more eggs and more eggs per mass than the Fauldhouse population despite the latter being larger at first reproduction. As reproductive output is generally a factor of adult size one can conclude either the Epping population is contributing disproportionately more to reproduction or the Fauldhouse population is contributing relatively less. The Epping population also displayed distinctions in shell morphology, weight to length ratios, and calcium content of the shells and it is likely that these traits may also be involved in the trade-off with juvenile survival and reproduction. The observed differences in this population relative to others seem to focus on traits related directly to the shell. As the main function of the gastropod shell is primarily defensive (Dillon, 2000) and higher calcium content in mollusc shells has been shown to increase crush resistance (Lewis and Magnuson, 1999) it is possible that the observed life history differences in the Epping population have been driven by shell crushing predators such as crayfish (Krist, 2002). The Epping population was shown to display consistency in many traits across generations (such as reproductive outputs, shell character and juvenile mortality) suggesting that the observed differences in life history described above result from a genetic component and that observed trade-offs in this population can be characterised as being microevolutionary in origin.

The Fauldhouse population was the most northerly of all the populations studied and was known to have been introduced in recent time to a habitat with low environmental calcium. This population displayed faster growth in the x10 replicate stage in the low calcium treatments in both generations (particularly in the F_2 generation) followed by slow growth rates in the linear phase of growth with lowest growth occurring in the low calcium treatment in the F_2 generation. Individuals from Fauldhouse were the largest of all populations at first reproduction with a marked trade-off in size vs. age at first reproduction becoming apparent only in low calcium treatments in the F_1 generation, suggesting that this population was particularly sensitive to

environmental calcium. This is supported by the fact that the F_2 generation was reluctant to self, with many individuals failing to reproduce by the end of the experiments and by a (non-significant) trend towards lower egg output in the low calcium treatments. Like the Epping population, Fauldhouse was shown to display higher reproductive output associated with higher size at maturity and little variation in weight to length ratio across calcium treatments. Support for an environmentally derived (physiological) trade-off further comes from the fact that the F_2 Fauldhouse population grew larger in low calcium treatments yet produced fewer eggs than the high calcium treatment group. This population is of interest as it appears to show a strong differential response to calcium across life stages that other populations did not. When reared in low calcium this was characterised by rapid growth in early life stages (although as this was in the x10 replicate stages this effect may be influenced by density (Brown, 1979a)) and slower growth to a larger size, with a corresponding increase in age at maturity. Given that this population may have been established by a relatively small number of individuals, it is possible that the strong differences across calcium treatment are derived from genetic effects relating to a population bottleneck (Coutellec and Caquet 2011) and colonisation history.

The intraspecific differences in life history and the trade-offs detected in this study serve as good evidence to support the first hypothesis. In general the pattern of high variability across the range of traits examined both between populations and between generations, and evidence for a combination of plasticity and genetic contributions is consistent with studies of other mollusc species (Chapuis et al., 2007, Pascoal et al., 2012). General differences in growth rates, shell weights and morphology across calcium treatments were consistent with a study by Rundle et al. (2004). However some populations (Fauldhouse and Epping) were shown to display little variation in shell weights across calcium treatments which would suggest that local adaptation influences plasticity in these traits. It would appear that local adaptation in the study populations is largely characterised by variation in traits associated with fitness such as size at first reproduction and reproductive output (Brown, 1983, Dillon, 2000) which appear to be under higher levels of genetic control

than other traits. Although there is clearly evidence of the role of calcium availability in determining life history variation, the relative lack of sensitivity of some traits would tend to indicate the potential role of other factors, such as predator identity, parasitism, and abundance or overall differences in productivity (Brown, 1985, Krist, 2002, Lewis and Magnuson, 1999, Rundle et al., 2004). When examined in terms of the combined effect of variation in life history traits on population growth through the application of matrix models to predict population growth rates, there was a relatively subtle response to calcium availability evident, along with limited variation between populations. When examined in more detail through elasticity analysis, it was clear that the variation in calcium availability had the largest effect on the early life stages (hatchlings and juveniles) as would be expected, leading to a greater proportional contribution by adult stages to overall population growth.

The second hypothesis was that adaptation to environmental conditions will influence the ability of populations to adapt to other stresses due to trade-offs in energy allocation. The populations that were chosen for more detailed comparison contrasted in the observed life history variation from the studies in Chapter 2.

Studies on the effects of acute exposure to nanoparticle CB in Chapter 4 revealed early life stages to be the most sensitive with greatest effects on mortality and growth being recorded in juvenile animals. Here a general dose-response relationship, with highest mortality recorded in highest CB concentrations was recorded. Similarly juvenile growth displayed a similar dose-response with lowest growth recorded in the highest CB concentrations. CB exposure was shown to cause up-regulation of reproduction, with higher reproductive output occurring in the highest CB concentrations (both in terms of the number of egg masses and number of eggs per individual). Such fecundity compensation may be a response to a perceived threat to current survival, resulting in greater investment in a ‘big bang’ reproductive strategy (Begon et al., 2006, Ranta et al., 2002) and has been shown to occur in other organisms in response to toxins (Jensen and Marshall, 1983). Such reproductive strategies are found more commonly in semelparous organisms

and it is possible that the responses recorded here may be reflective of the evolutionary history of *L. stagnalis* which is thought to have evolved an iteroparous life cycle from a semelparous state more common to pulmonates (McMahon, 1983). This observation in conjunction with the retention of trade-offs in reproductive traits described with respect to hypothesis 1 would indicate that *L. stagnalis* retains some characteristics more commonly associated with semelparous reproductive strategies.

The chronic exposure studies revealed clear evidence of intraspecific differences in response to CB across calcium regimes, with population differences only becoming apparent in the low calcium treatments. Elasticity analyses revealed that the Bank Well and Savernake populations displayed a similar response in the low calcium exposures, with greater sensitivity in early life stages prompting a shift toward a higher contribution from the adult stages to population growth. The Fauldhouse population was shown to be unaffected by CB exposure in either calcium regime. In the life history study (Chapter 2) this population was shown to display phenotypic plasticity in the majority of life history traits in response to environmental calcium. However, the lack of any correlation of responses across generations would indicate that the major component of this variation is environmental rather than genetic for most traits. However, hatchling growth rates were shown to be consistently higher in low calcium (markedly so in the F_2 generation) suggesting that early life stages fair better in low calcium regimes. This could suggest that the Fauldhouse population is pre adapted to a low calcium environment and may help to explain the lack of response at the population level to CB in the chronic exposure. Further support for this view comes from the fact that the Fauldhouse population was known to be introduced, displaying a marked reluctance to self in the F_2 low calcium treatment, suggesting that this population may have previously been subject to high levels of inbreeding, possibly due to founder effects at introduction (Coutellec-Vreto et al., 1998). Thus, the lack of response to CB NPs in juvenile survivorship in this population could be derived from effects associated with a lack of genetic variability and fixation of traits involved in response to

environmental calcium derived from inbreeding in this population (Coutellec and Caquet, 2011).

In Chapter 4 a marked reduction in population growth rate (lambda) was shown in the low calcium treatment for the Savernake and Bank Well populations while no difference in lambda was observed in the Faudhouse population. The Savernake and Bank Well population's lambda values suggested that they would not persist as natural populations, particularly as factors such as predation and inter specific competition, which would likely depress population growth rates further, were not considered in the matrix models. However, it is difficult to relate the findings of this study to actual effects in the field.

It is possible that the experimental conditions (in the form of cup volumes) in the chronic study may have resulted in a failure to detect fecundity compensation that was evident in the acute studies. This resulted in the use of fecundity data derived from non-CB exposed adults from the F_2 study and it is possible that the effects on fecundity have been underestimated, although defence for this omission comes from the fact that fecundity compensation was only recorded at higher carbon black concentrations (10 and 100mgL^{-1}) than those used in this study. It is also possible that this effect was only specific to the Union Canal population that was used in the acute exposure, however further work carried out in an undergraduate student project (not presented here) suggests that the Fauldhouse population also display fecundity compensation when exposed to 100mgL^{-1} CB.

In summary the results of this study present strong support for the second hypothesis. Significant intraspecific differences in response to CB NP stress only became apparent in low calcium treatments in the Savernake and Bank Well populations while the Fauldhouse population displayed little response. These differences are suggestive of local adaptation to different levels of calcium which, due to the adoption of different life history strategies, result in markedly different responses to the same stressor across populations. This is consistent with other recent studies that have recorded important differences

in responses of other organisms depending on population source or genetic variation (Ducrot et al., 2010, Salice et al., 2009) and also the environmental context in which they are exposed to stressors (Paul-Pont et al., 2010, Coors et al., 2004, Duchet et al., 2010, Hanson, 2011).

Limitations and further studies:

It is possible that the calcium concentrations used in this study did not sufficiently challenge the organisms, resulting in the slightly muted responses shown in some parts of the work. Analysis of the sites where the populations were sampled indicated that prevailing calcium levels were lower than those recorded in the data that was used to determine which sites to sample in many cases. Despite being classified as a calciphile species (Kerney, 1999, Boycott, 1936, Briers, 2003), *L.stagnalis* is clearly able to maintain populations in conditions substantially below the supposed 'cut-off' of 20mgL^{-1}. By using a calcium concentration below 40mgL^{-1}, future studies may detect differences in traits that were not observed in these studies.

Life histories over a geographic range are likely to be shaped by a number of other factors such as predation (Krist, 2002, Lewis, 2001), temperature (Brown, 1979a), parasitism (Sorensen and Minchella, 1998) and a number of other factors that were not considered in this study. Further studies could therefore focus on combined effects of environmental stressors in shaping life histories across a geographic range. Given the considerable costs of investment in the shell and that this structure is primarily defensive (Dillon, 2000), it is likely that explorations of the relationship between life history variation, predation risks and environmental calcium may prove fruitful.

Summary of research findings:

This study revealed high levels of variation in life history traits across the study populations. Phenotypic plasticity, derived from a mixture of environmental and genetic factors, was detected in all traits with respect to calcium treatment but the nature and magnitude of response was shown to vary between populations and conserved responses to environmental calcium tended to be less readily detected. Conserved differences tended to be more

manifest in traits related to somatic growth and became more pronounced in the F_2 generation once environmental effects had been removed. It is possible that this is influenced by the fact that the low calcium concentration used in this study was not sufficiently low to challenge the organisms. Intraspecific differences in life history strategies were characterised by variation in life history traits which appeared to be more related to fitness, which also tended to display low heritabilities. In particular size at first reproduction, which was shown to be directly linked to reproductive output, (and trade-offs therein) was shown to vary across the study populations. Variation in this trait was shown to be under strong genetic control and displayed low heritability suggesting that size at first reproduction, and correlated reproductive traits, are most important in determining life history strategy across the distribution sampled.

Environmental calcium was shown to have a subtle effect on population growth rates by the F_2 generation but this was not found to be significant. The effects of inbreeding due to selfing were shown to have a far greater depression on population growth rate. Elasticity analysis revealed that the significant reduction in lambda due to inbreeding and the more subtle effects derived from calcium, resulted from a greater contribution from adult stages to population growth due to higher juvenile mortality. This suggests that a greater contribution from adult stages to population growth is a common response to stress.

Local adaptation to calcium availability was shown to influence the life history response to nanoparticulate carbon black, and was mirrored in predicted population growth rates obtained from matrix models. Intraspecific differences in response to carbon black nanoparticles only became apparent when calcium concentrations were low. These findings would support the view that in order to be better able to predict the response of species to the presence of novel stressors such as nanoparticles, it is necessary to account for intraspecific adaptation of life history traits as well as geographical variation in the environmental context.

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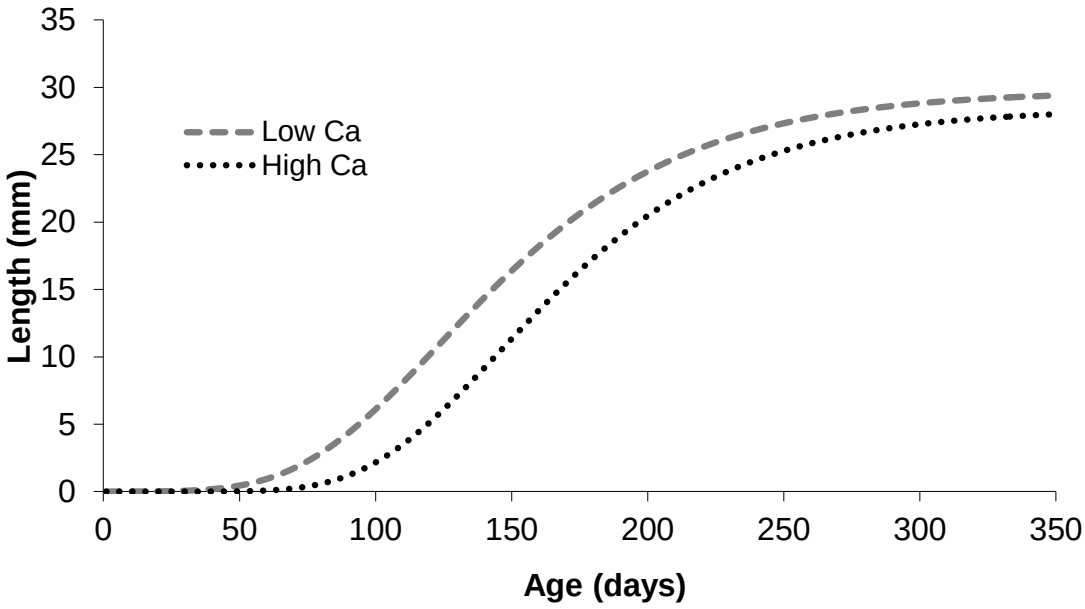
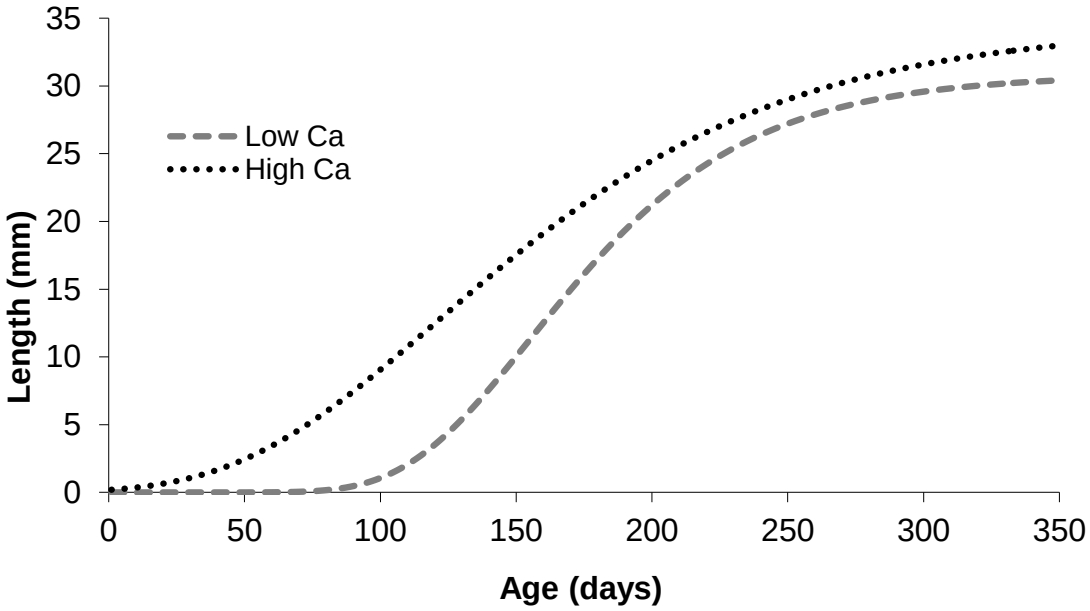
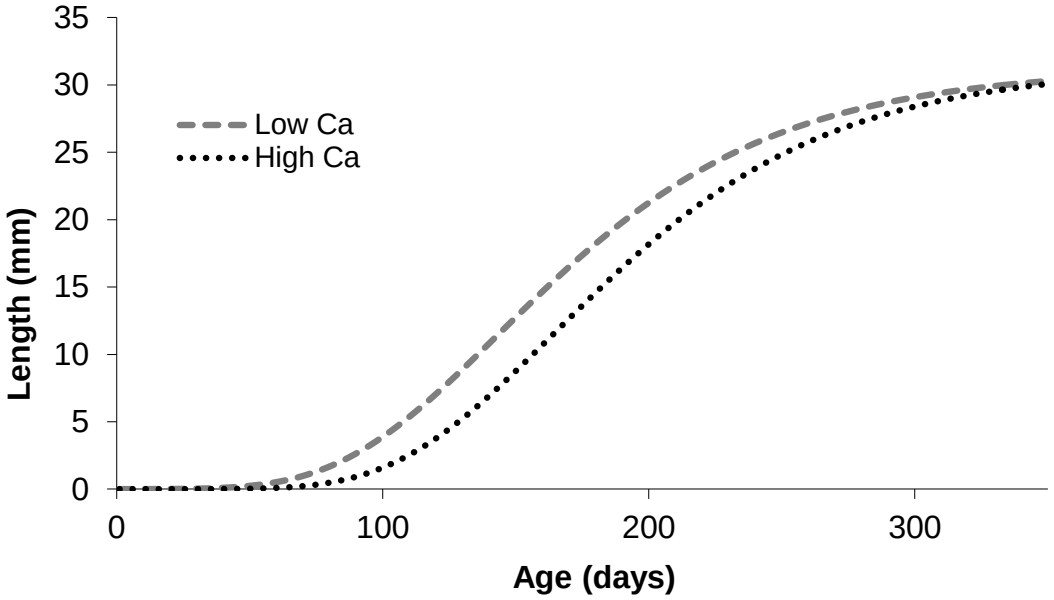
7. Appendices



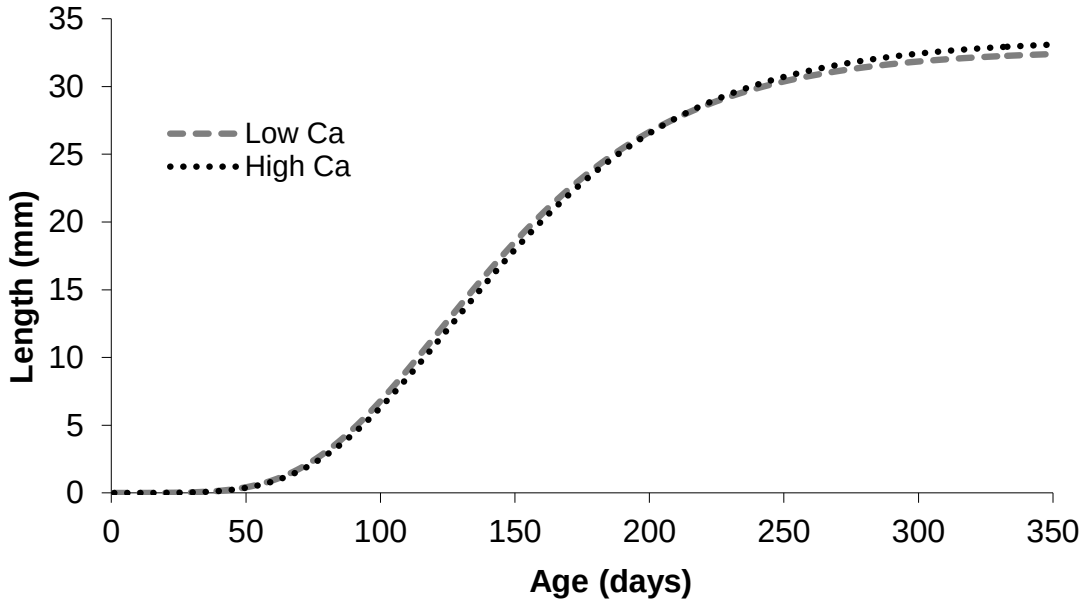
Appendix 6.1. Gompertz model growth curve for Bank Well F_1 generation high and low calcium.



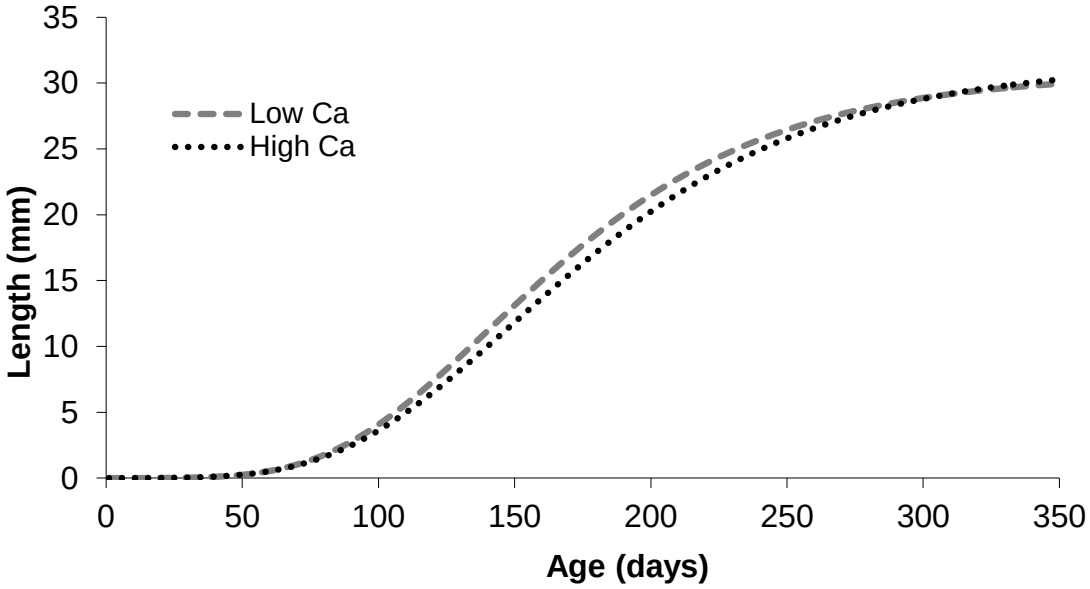
Appendix 6.2. Gompertz model growth curve for Brychfa F_1 generation high and low calcium.



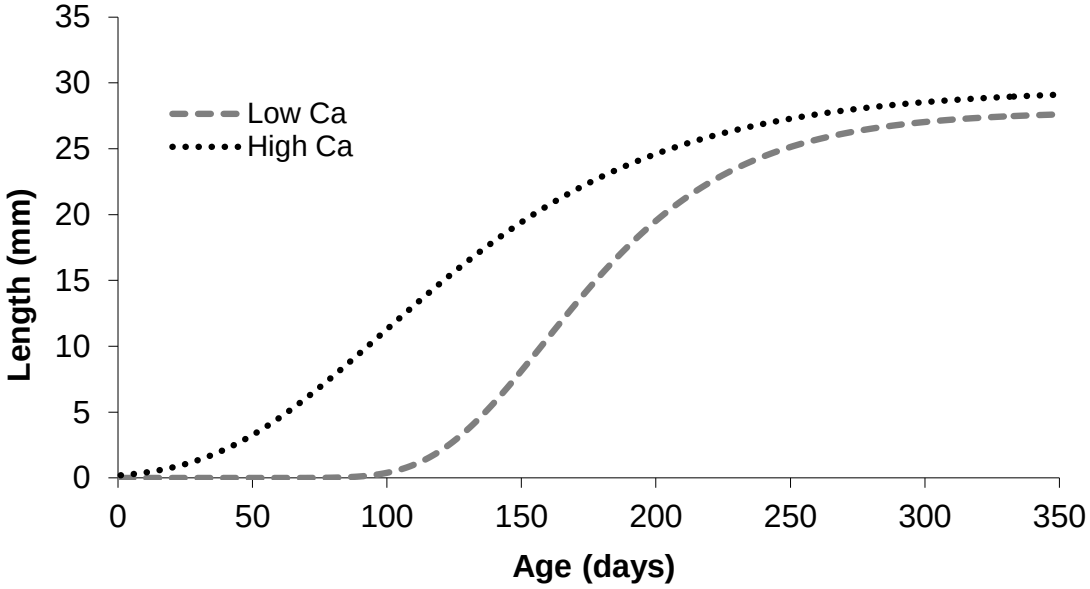
Appendix 6.3. Gompertz model growth curve for Epping F_1 generation high and low calcium.



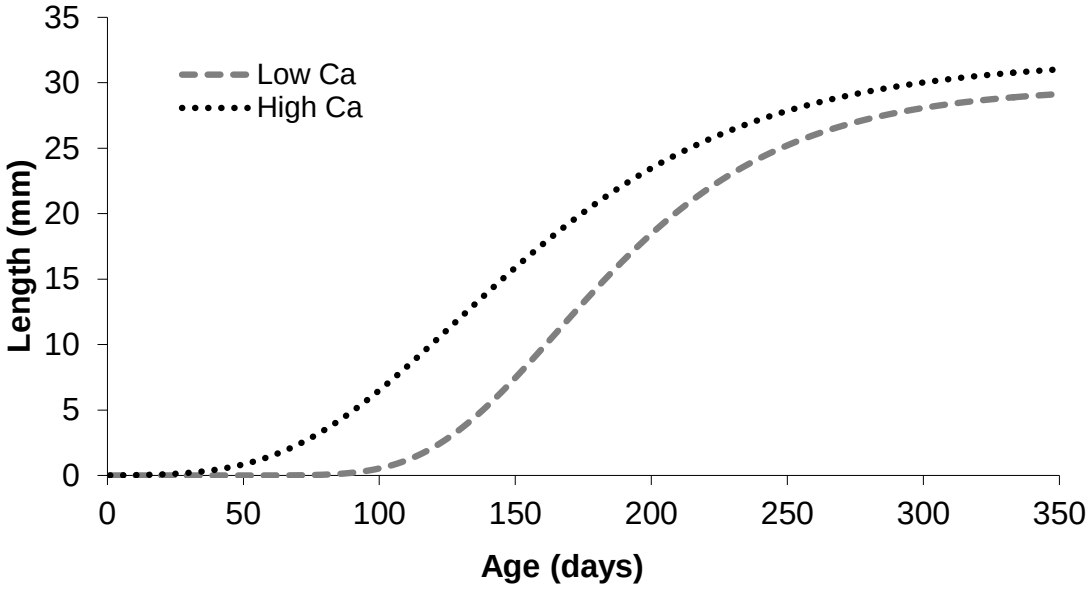
Appendix 6.4. Gompertz model growth curve for Fauldhouse F_1 generation high and low calcium.



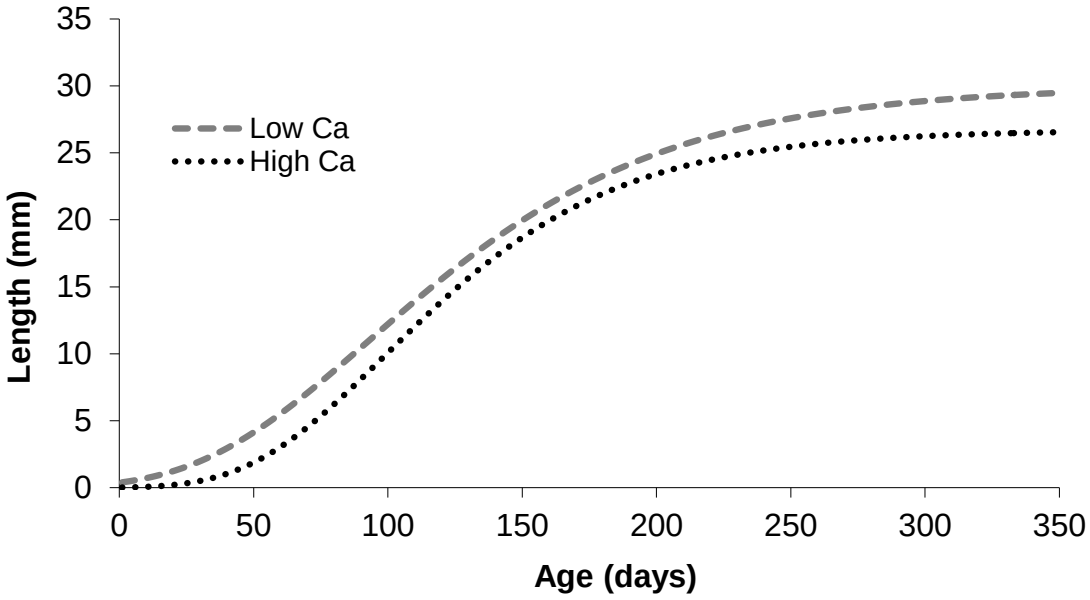
Appendix 6.5. Gompertz model growth curve for Glanahafren F_1 generation high and low calcium.



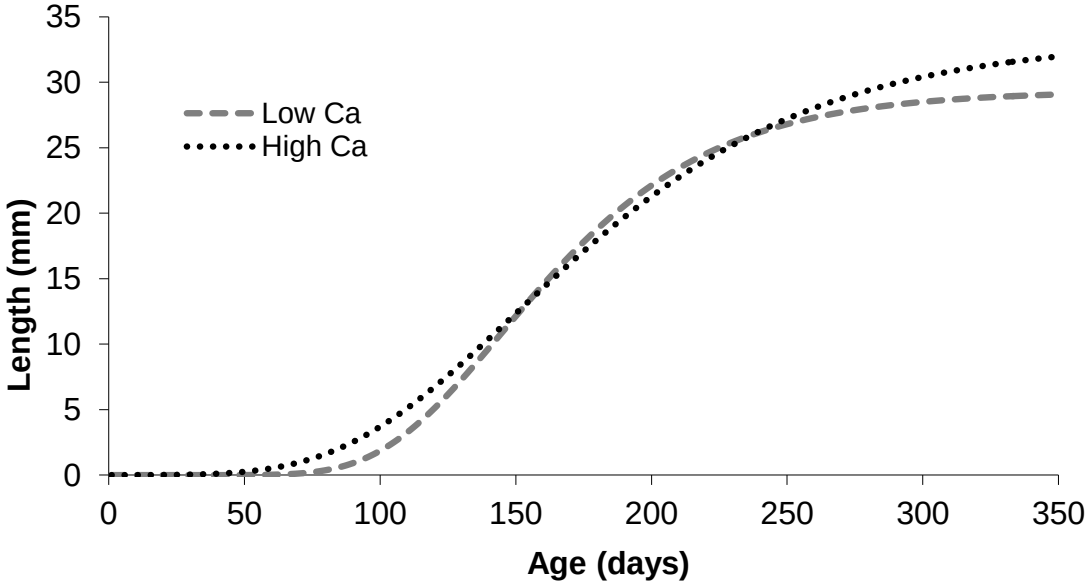
Appendix 6.6. Gompertz model growth curve for Ireland F_1 generation high and low calcium.



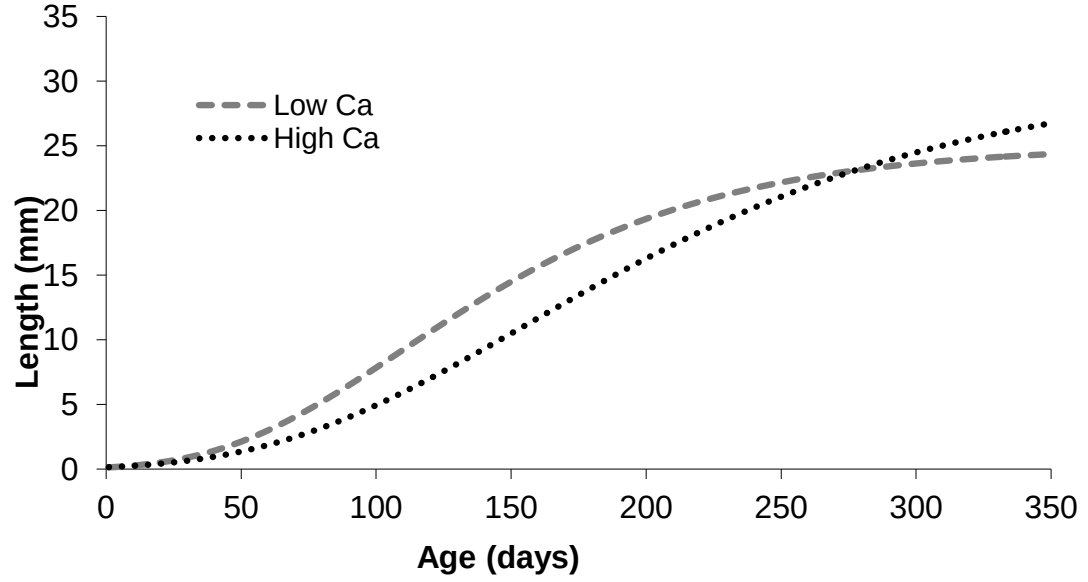
Appendix 6.7. Gompertz model growth curve for Savernake F_1 generation high and low calcium.



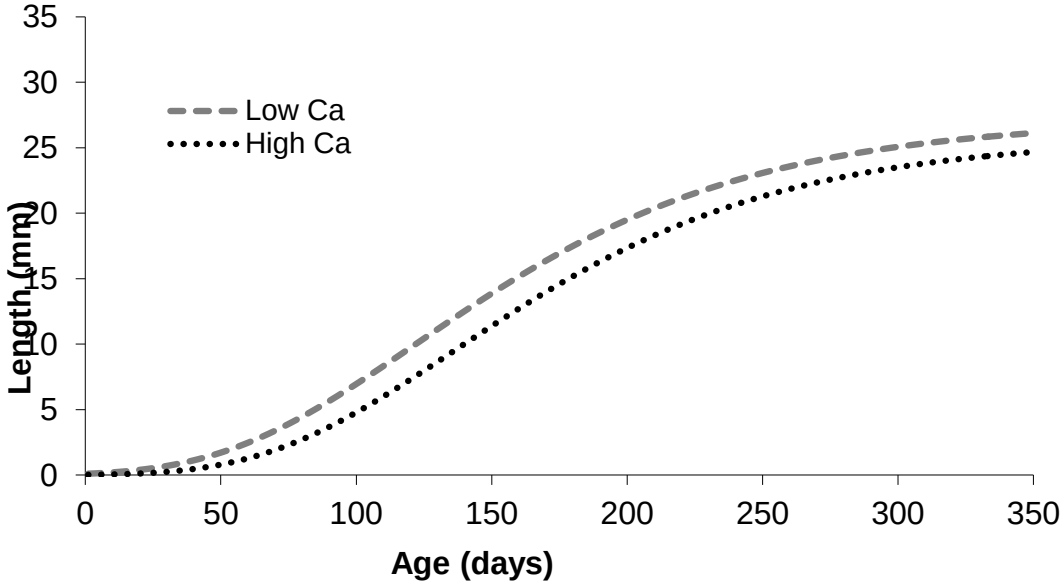
Appendix 6.8. Gompertz model growth curve for Tiv F_1 generation high and low calcium.



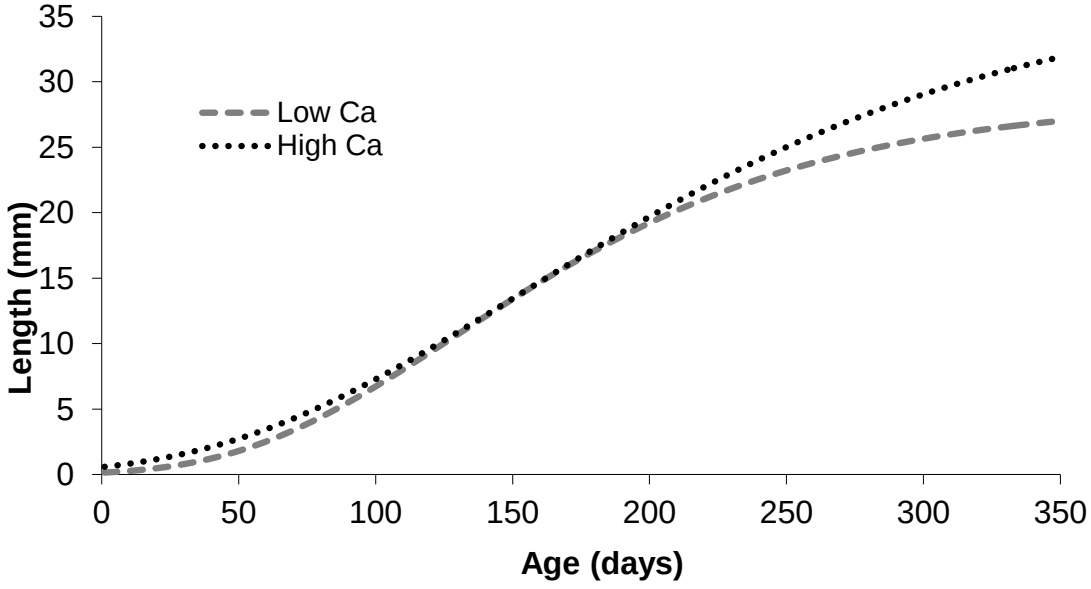
Appendix 6.9. Gompertz model growth curve for Wreake F_1 generation high and low calcium.



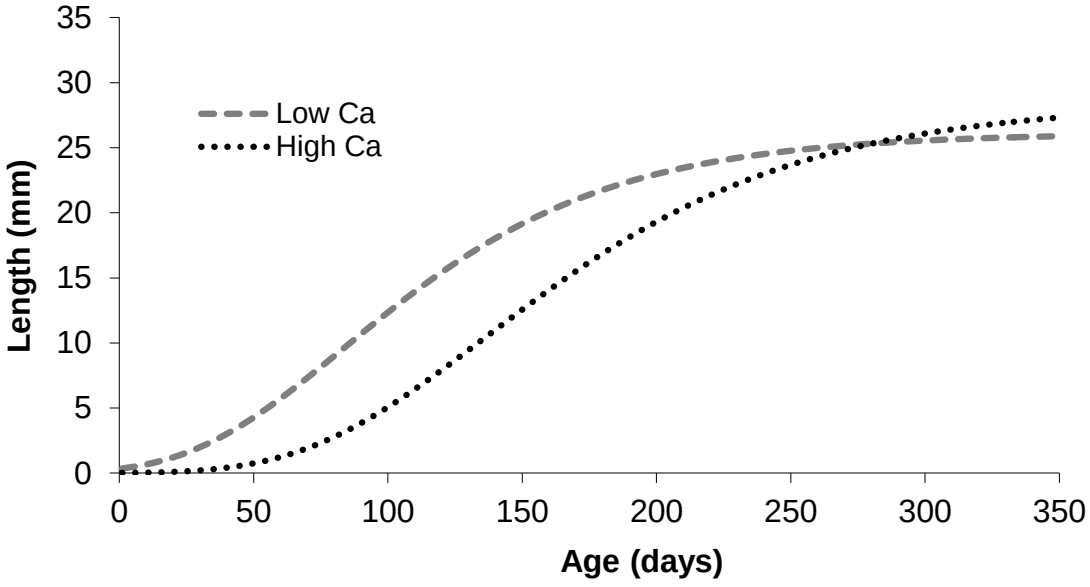
Appendix 6.10. Gompertz model growth curve for Bank F_2 generation high and low calcium.



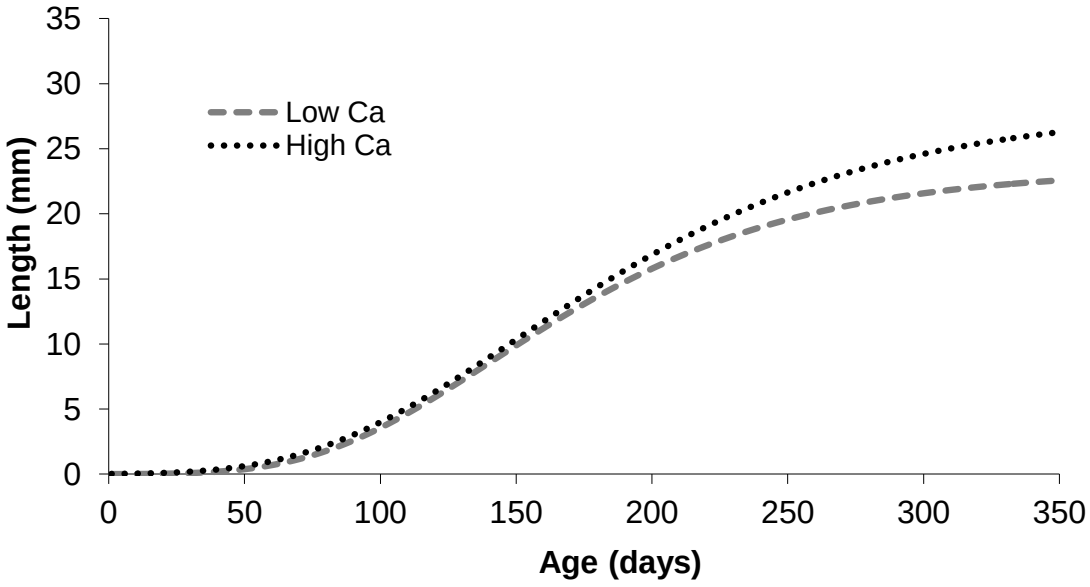
Appendix 6.11. Gompertz model growth curve for Brychfa F_2 generation high and low calcium.



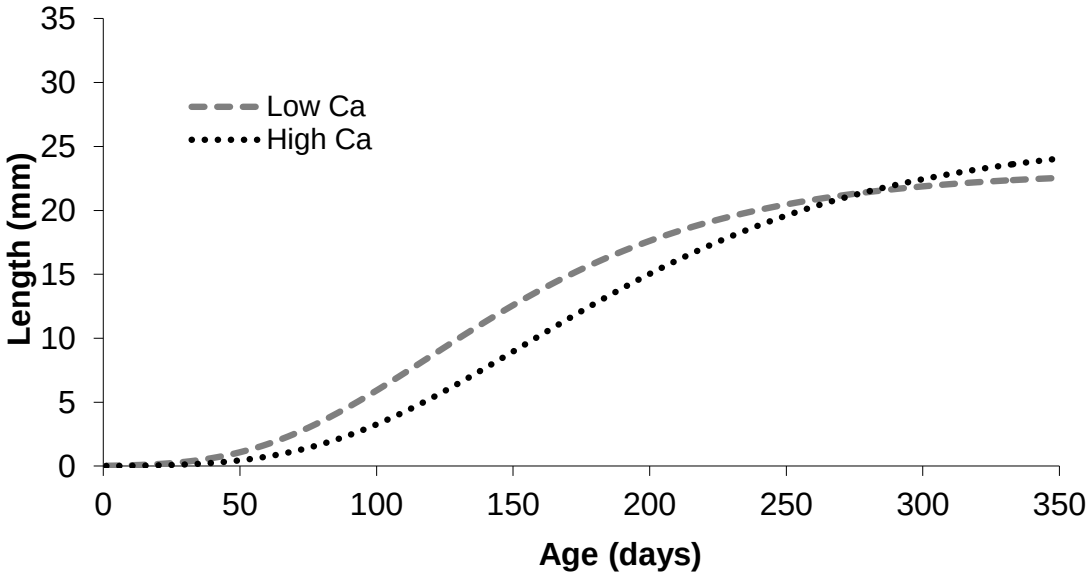
Appendix 6.12. Gompertz model growth curve for Epping F_2 generation high and low calcium.



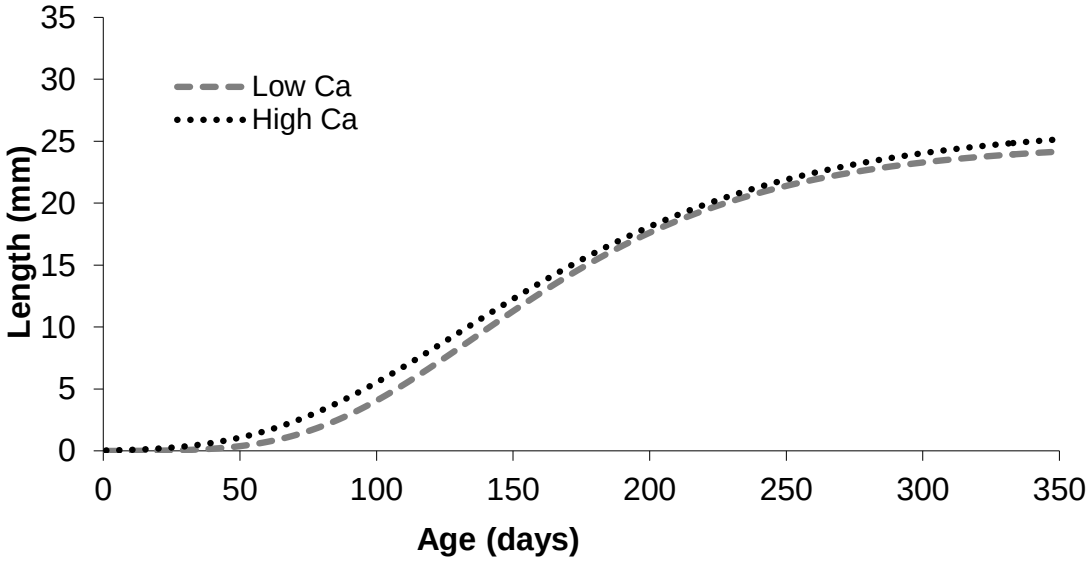
Appendix 6.13. Gompertz model growth curve for Fauldhouse F_2 generation high and low calcium.



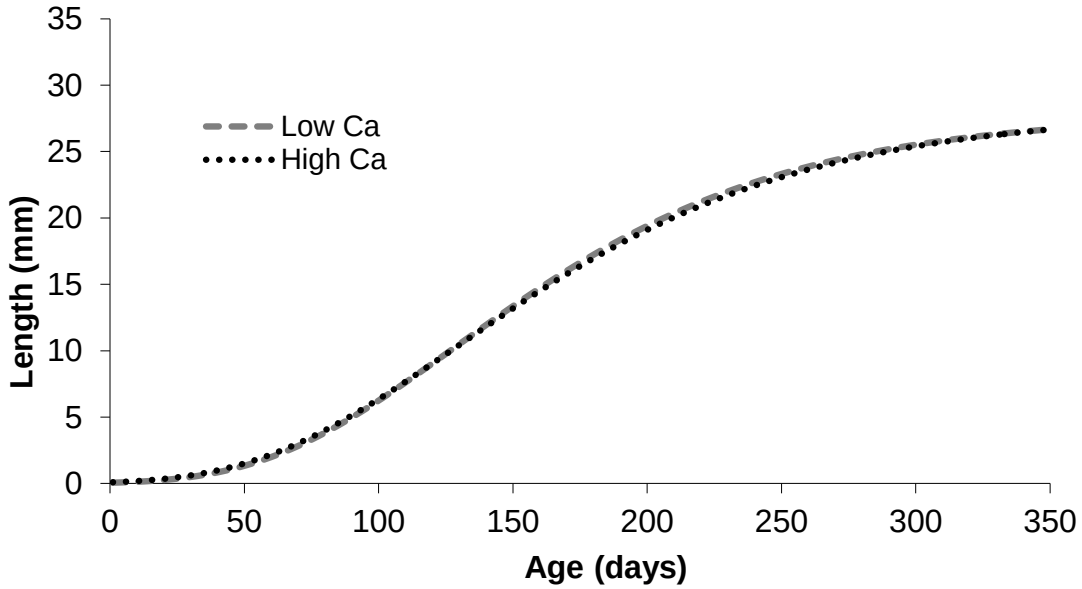
Appendix 6.14. Gompertz model growth curve for Glanahafren F_2 generation high and low calcium.



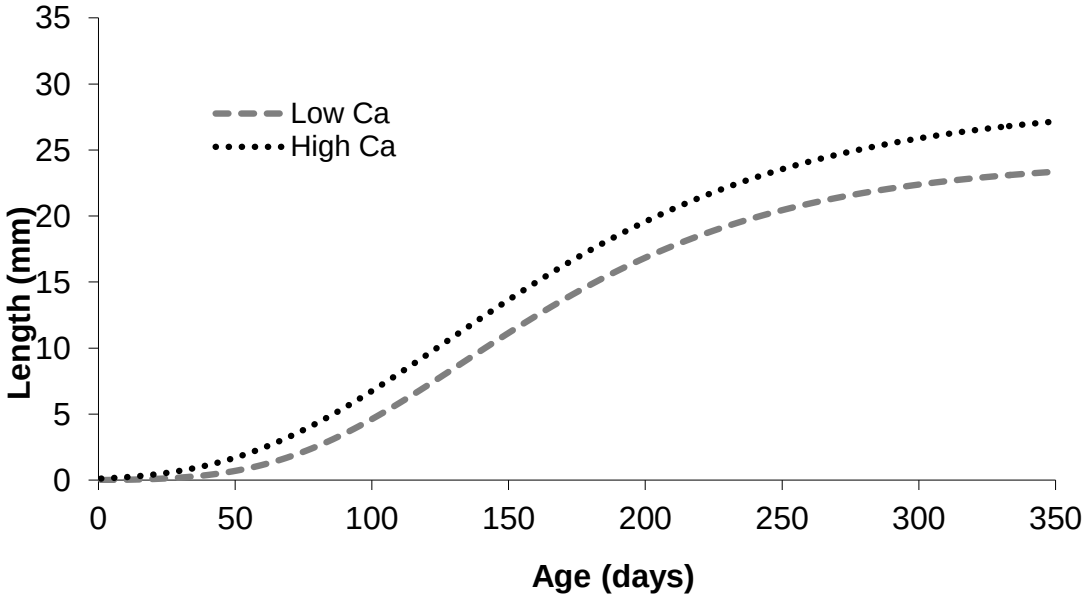
Appendix 6.15. Gompertz model growth curve for Ireland F_2 generation high and low calcium.



Appendix 6.16. Gompertz model growth curve for Savernake F_2 generation high and low calcium.



Appendix 6.17. Gompertz model growth curve for Tiverton F_2 generation high and low calcium.



Appendix 6.18. Gompertz model growth curve for Wreake F_2 generation high and low calcium.