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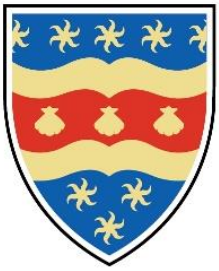

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Do abiotic environmental drivers disrupt the biotic response of marine larvae?

by

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Author's Declaration

At no time during the registration for the degree of Research Masters has the author been registered for any other University award without prior agreement of the Doctoral College Quality Sub-Committee.

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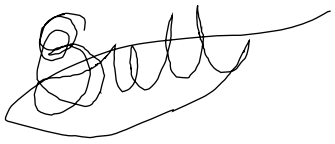

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Stanley Edward Butt

Do Abiotic Environmental Drivers Disrupt the Biotic response of Marine Larvae?

Abstract

Phenotypic plasticity, the ability of a given genotype to express different phenotypes in response to altered environmental conditions has been identified as a means by which organisms may be able to cope with environmental change. While significant progress has been made in the study of phenotypic plasticity in response to both individual and combined abiotic environmental drivers, very few studies have investigated the effects of abiotic and biotic drivers in combination. In this thesis, I investigate the combined effects of increased temperature (15 °C versus 20 °C) and the presence of predation threat in the form of predatory kairomones (skin mucus of predatory fish) on the embryonic development and subsequent locomotion of hatchling veliger larvae of the marine gastropod *L. littorea*. There were significant interactive effects of developmental exposure to predator kairomones and increased temperature. At 15 °C, exposure to predator kairomone led to accelerated development, whereas at 20 °C they retarded development. Predator kairomones also influenced the morphology and swimming of hatched veliger larvae. Larvae that were exposed developmentally to predator kairomones had significantly larger shells (20 %) and velums (22 %), and swimming was significantly faster, regardless of temperature. Veliger swimming speed was also positively correlated with velum size for those developed under predator presence, but no such relationship was present in control treatment larvae.

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This study demonstrates that abiotic and biotic environmental drivers may lead to complex responses in marine larvae, including carry-over effects between life history stages. Such effects may have ecological implications due to the alteration in the relationships between predators and prey in projected climate change conditions. This study also explores how climate drivers and predation threat interact on the development rates and morphology of *L. littorea*, and trade-offs between accelerated development and increased morphological size at hatch. The trade-off may have implications on further developed *L. littorea*, and would need further investigation to explore this interaction up to and exceeding the settlement stages.

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Chapter One: Phenotypic plasticity in marine invertebrates in response to global environmental change

1.1 Introduction

Phenotypic plasticity is the ability of an individual genotype to express multiple, environmentally-dependent phenotypes (Agrawal, 2001) and has been demonstrated in a wide range of organisms (DeWitt and Scheiner, 2004; Palmer, Bush and Maloof, 2012; Padilla and Savedo, 2013). It has been suggested that phenotypic plasticity can play a major role in the ability of an organism to adapt to an environment (Agrawal, 2001; Hoverman and Relyea, 2009), and is predicted to be high for those species that experience heterogenous environments (Agrawal, 2001; Van Buskirk, 2002). Plasticity can be defined as either active, or passive (Whitman and Agrawal, 2009). Active plasticity is a coordinated, complex change that includes multiple regulatory genes, whereas passive plasticity is usually a result of physical or chemical environmental stressors that directly alter physiological processes, such as a small size as a result of poor nutrition (Whitman and Agrawal, 2009), or as a response to extremes in the environment such as climate change. Anthropogenically induced climate change is leading to rapid environmental change globally and this is particularly pronounced in heterogeneous marine environments (Doney *et al.*, 2012). Chapter 1 reviews our understanding of how marine invertebrates, a taxonomically and physiologically diverse group, respond to environmental change using phenotypic plasticity. Marine invertebrates have proven an important group for the study of phenotypic plasticity in traits ranging from predator avoidance and swimming behaviours, to the size of feeding appendages and increased spine size in response to both abiotic and biotic drivers

(Agrawal, 2001; Li and Denny, 2004; Claireaux, Couturier and Groison, 2006; Whitman and Agrawal, 2009; Chevin, Lande and Mace, 2010; Buskey, Lenz and Hartline, 2012; Charpentier, Wright and Cohen, 2017). Marine invertebrate life histories are also extremely diverse and therefore this literature review incorporates phenotypic plasticity across the breadth of life history stages including larval stages. With over 15 marine phyla producing larvae (Strathmann, 1993), early life history stages are particularly important in understanding the response of marine organisms to projected climate change (Hoegh-Guldberg and Pearse, 1995; Przeslawski, Byrne and Mellin, 2015).

Temperature is one of the key determinants of the phenotype (Iverson *et al.*, 2020) and is one of the primary abiotic stressors associated with climate change (Barrows *et al.*, 2007; Thompson, 2010), with increased air temperature directly influencing sea surface temperatures (Barrows *et al.*, 2007), inclusive of coastal systems (Harley *et al.*, 2006). Sea surface temperatures have a projected increase of 1 – 4 °C by 2100 (Laffoley and Baxter, 2016). Study of the wide-ranging impacts of temperature on the biochemistry, development and ultimately the fitness of organisms has a rich history in biology (Robinson, Peters and Zimmermann, 1983; Hoegh-Guldberg and Pearse, 1995; Oliphant, Hauton and Thatje, 2013), with studies from 1915 proposing Universal Temperature Dependence (UTD), but which was later described as an oversimplification of biological processes (Arrhenius, 1915; Knies and Kingsolver, 2010; Mundim *et al.*, 2020). Due to the multifaceted nature of anthropogenic climate change and the complexity of biological and ecological processes, understanding the impact of temperature with other environmental drivers is an important step for understanding the response of organisms to future environmental conditions (Przeslawski, Byrne and Mellin, 2015). Predation is an important environmental driver to consider as almost all

organisms experience predation threat at some stage of their life cycle, and threat can be specific to particular life stages (Brönmark and Miner, 1992; Cowan, Houde and Rose, 1996; Tollrian and Laforsch, 2006; Buskey, Lenz and Hartline, 2012). Therefore, to gain a better understanding of how these abiotic and biotic drivers will interact with one another in marine invertebrate larvae, this review will focus on the study of phenotypic plasticity in response to environmental drivers associated with global climate change, in combination with predation, one of the most important ecological biotic drivers (Coors and Meester, 2008; Chevin, Lande and Mace, 2010; Dixson, Munday and Jones, 2010).

1.2 Phenotypic plasticity

The observable characteristics of an organism are its phenotypes, with the genetic precursor to phenotypes being the genotype (Orgogozo, Morizot and Martin, 2015). The match between the phenotype and the environment determines the fitness of a population; however, for species that experience heterogenous environments, a fixed phenotypic trait will not allow for maximum fitness (Auld, Agrawal and Relyea, 2010). This is where phenotypic plasticity comes in, enabling the phenotype to change in response to the environment. Phenotypic plasticity has been acknowledged by botanists far longer (Bradshaw, 2006) than it has been studied in the animal kingdom (Sommer, 2020), and a growing awareness of phenotypic plasticity occurred from the late 20th century (Sommer, 2020); however, the first writings on the subject date back to the end of the 19th century (Baldwin, 1896; Fusco and Minelli, 2010), with those investigating what was known as the “Reaktionsnorm” (norm of reaction) to explain how developmental responses to the environment may occur and evolve (Gabriel and Lynch, 1992).

Phenotypic plasticity can relate to traits that are behavioural, chemical, mechanical or morphological (Ghalambor *et al.*, 2007), and is likely a result of variation and fluctuation in the environment (Nijhout, 2003). Fluctuations in the environment can be either biotic or abiotic, and can be a result of seasonal variation, human interference or non-native species introduction, that lead to differences in environmental drivers including light availability, temperature, and food availability (Pelletier *et al.*, 2007; Kingsolver and Buckley, 2017). These fluctuations can mean that a stationary phenotype may experience a reduction in fitness when subject to a variable environment, as a phenotype that is optimal in one set of environmental conditions is likely to be sub-optimal in another set of environmental conditions (Pelletier *et al.*, 2007; Auld, Agrawal and Relyea, 2010). Consequently, phenotypic plasticity has evolved to allow for a shift in the optimum phenotypes in an attempt to better suit the variable environmental conditions (Pelletier *et al.*, 2007; Auld, Agrawal and Relyea, 2010), yet there may be costs and limits associated with these phenotypically plastic traits (DeWitt, Sih and Wilson, 1998; Auld, Agrawal and Relyea, 2010).

Costs to plasticity were defined by DeWitt, Sih and Wilson (1998) as where the fitness of a plastic phenotype is lower than that for a fixed phenotype in a focal environment. They include production and maintenance costs, information acquisition costs, developmental instability and 'genetic costs'. Limits set out by DeWitt, Sih and Wilson (1998) are where a plastic phenotype is unable to produce the same (optimal) phenotype as a fixed one. They include lag-time limit, information reliability, developmental range limit and the epiphenotype problem.

Phenotypic plasticity can be energetically costly due to increased sensory regulation and the morphosis of plastic traits, whilst also maintaining regular

physiological processes. Auld, Agrawal and Relyea (2010) describe that production and maintenance costs are fundamentally distinct from one another, as maintenance costs are environmentally-independent, and production costs are environmentally-dependant. Maintenance costs are environmentally-independent because organisms use their physiological mechanisms regardless of the environment, whereas producing a physiological response depends on the environment in which the organism is subject to, hence it is environmentally-dependant (Scheiner and Berrigan, 1998; Sultan and Spencer, 2002; Auld, Agrawal and Relyea, 2010).

Information acquisition allows an organism to detect changes in the environment to be able to express phenotypically plastic traits DeWitt, Sih and Wilson, 1998), and is potentially costly as gathering information may be additionally energetic and reduce foraging or mating efficiency (DeWitt, Sih and Wilson, 1998; Auld, Agrawal and Relyea, 2010). Auld, Agrawal and Relyea (2010) note that information acquisition costs should be a factor regardless of the environment, as well as stating that developmental costs are not true costs of plasticity. Developmental instability is the costs associated with poor environment matching for the individual from being subject to environmentally sensitive processes during development (DeWitt, Sih and Wilson, 1998; Auld, Agrawal and Relyea, 2010); however, this is seen more as an phenotype-environment mismatch and not as a cost to plasticity (Auld, Agrawal and Relyea, 2010).

It has been suggested that the genetic costs defined in the review by DeWitt, Sih and Wilson (1998) should be defined as 'intrinsic genetic costs' (van Kleunen and Fischer, 2005). This would incorporate pleiotropy and epistasis, both plastic responses that are in direct relationship with loci that affect plasticity, or with negative fitness effects (Auld, Agrawal and Relyea, 2010)**.** Loci are a particular set of genes or genetic markers that are

in a fixed position on a chromosome, one locus will have multiple variations of itself, known as alleles (Elston, Satagopan and Sun, 2012). Epistasis is suggested to be where plastic traits alter the expression of other traits indirectly (DeWitt, Sih and Wilson, 1998). Both of these 'intrinsic genetic costs' represent negative correlations between plasticity and fitness (van Kleunen and Fischer, 2005). DeWitt, Sih and Wilson (1998) discuss pleiotropy, where the formation or alteration of phenotypic responses may have a significant effect on other non-plastic phenotypes as a result of associated altered gene expression. Mills, Greenwood and Peichel (2014) demonstrate that pleiotropy is evident between the lateral plates and the location of the neuromasts along the lateral line; therefore, if a driver were to influence the lateral plates, it would affect the location of the neuromasts as well.

Included in the limits set out by DeWitt, Sih and Wilson (1998) is lag-time, the time between the environmental change and the response an organism has resulting from this change. Behavioural traits may not take long after exposure to express different behaviour, it may only take a few minutes (Magnhagen and Forsgren, 1991; Mery and Burns, 2010; Bhat, Greulich and Martins, 2015). This lag-time means that costs may be limited to the individuals, but physiological processes can take much longer, especially in processes involving morphological change. The rate and duration of developing morphological structures determine the lag-time for that organism, which could take days for some organisms during early stages of development (Vaughn and Strathmann, 2008; Fusco and Minelli, 2010; Charpentier, Wright and Cohen, 2017), but morphological responses are likely to be much slower than during adult life stages (Li and Denny, 2004; Bibby *et al.*, 2007).

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When DeWitt, Sih and Wilson (1998) discuss the limits to plasticity, one area which they investigate is the reliability of the information collected, as the sensory organs may collect incorrect information from the environment or be limited in sensitivity. Information reliability is fundamental to the production of environment-matching phenotypically plastic traits (Auld, Agrawal and Relyea, 2010) as poor reliability of the information collected may result in a plastic trait poorly matched to the environment. One possibility for a reduction in information reliability may result from collecting information from a combination of factors from the surrounding environment (Breitburg *et al.*, 2015). Some studies show that ocean acidification, the increase of pCO_2 in the ocean from anthropogenic climate change, interferes with the reception of other chemical cues, including predator cues (Munday *et al.*, 2009; Dixson, Munday and Jones, 2010; Cripps, Munday and McCormick, 2011). Anemone fish ordinarily avoid predation threat, but when exposed to ocean acidification this response is no longer present. The anemone fish respond to predator cues by expressing an attraction and swimming towards the source of the cues, perceiving them as non-threatening (Dixson, Munday and Jones, 2010). This inhibition of predator detection through chemoreception demonstrates that the exposure to lowered pH interacts with the perception of data received by the chemoreceptors, which negatively alters behaviour patterns; showing that the information received by the anemone fish was unreliable and led to maladaptive behaviour. Auld, Agrawal and Relyea (2010) state that both lag-time and information-reliability originate from the poor ability to detect and/or respond to environmental drivers, concluding that the mechanisms that allow for the gathering of information and maintenance costs of the sensors underpin the phenotypically plastic response.

Developmental range as a limit looks into how plastic a phenotype can be, suggesting that some canalized phenotypes may produce more extreme traits compared to those that express phenotypic plasticity (DeWitt, Sih and Wilson, 1998; Auld, Agrawal and Relyea, 2010). Canalization is when an organism expresses one phenotype that fits the heterogenous environment; therefore, when an organism expresses canalized traits, it is well-suited to one particular environment but can still function in sub-optimal conditions (Pigliucci, 2005; Auld, Agrawal and Relyea, 2010). The developmental range of an organism requires information about the environment, and the development of more extreme-fitting phenotypes may be limited due to production or maintenance costs (Auld, Agrawal and Relyea, 2010); therefore, the idea that developmental range is a limit to plasticity was rejected by van Kleunen and Fischer (2005), as it was proposed that it is a result of production and maintenance costs.

Epiphenotype problem, proposed by DeWitt, Sih and Wilson (1998), is when detection of an environmental stressor occurs late during development and the organism can't express adequate phenotypically plastic traits in response (Auld, Agrawal and Relyea, 2010). This suggests that trait changes early on during development are irreversible (Auld, Agrawal and Relyea, 2010); however, it has been shown that the timing of exposure to environmental drivers during development impacts the ability for an organism to respond (Relyea, 2003; Moore and Martin, 2019).

Whitman and Agrawal (2009) proposed that phenotypic plasticity is universal among living things as a response to a changing environment, as phenotypes that do not fit the environment lower fitness due to destabilised homeostasis and development. Research has demonstrated that the expression of phenotypic plasticity can increase survival in habitats complimentary to the phenotype expressed (Thompson, 1991;

Chevin, Lande and Mace, 2010). This is termed adaptive plasticity - situations in which the plastic response is beneficial for the organism (Nijhout, 2003; Ghalambor *et al.*, 2007). It has also been observed that some phenotypic plasticity has the ability to reduce fitness, and the cost of expressing these phenotypes is higher than not expressing them (Ghalambor *et al.*, 2007). An example of this can be found when looking at the Sydney Rock Oyster, *Saccostrea glomerata*, which expresses a larger size when subject to an increase in pCO_2 (Parker *et al.*, 2017). This response to pCO_2 proved maladaptive, as parental exposure under multistressor treatments led to offspring with significantly lower survival rates. Non-adaptive, or maldaptive, plasticity moves the organism further away from this new optimum (Morris and Rogers, 2013). The environmental drivers result in a shift to what would be the optimum for organisms, and the maladaptive phenotypically plastic response reduces the fitness of an organism and places the organism further away from this shifted optimum (Ghalambor *et al.*, 2007).

Until the late 1980s phenotypic plasticity was perceived as a barrier to evolution by natural selection (West-Eberhard, 2005; Fitzpatrick, 2012), with Wright (1931) stating that there is overwhelming evidence against an organisms physiological response to an environment being transmissible to later generations. However, the evolution of phenotypic plasticity itself is now an active area of research (Thompson, 1991; Pigliucci, 2005; Fitzpatrick, 2012). It is thought that the ability to remain phenotypically plastic allows organisms to adapt to unfavourable environments (Chevin, Lande and Mace, 2010), and has also been shown to impact the adaptation of a species via the heritability of plastic traits (Touchon and Robertson, 2018), suggesting that phenotypic plasticity has a role in evolution through adaptation (Chevin, Lande and Mace, 2010; Pfennig *et al.*, 2010; Dayan, Crawford and Oleksiak, 2015; Kelly *et al.*, 2017). Studies have shown

that these plastic traits may provide a mechanism for adaptations to evolve over shorter timescales (Ghalambor *et al.*, 2007). Agrawal (2001) shows that phenotypic plasticity may facilitate evolution by allowing organisms to adapt to variable abiotic or biotic conditions, which allows for colonisation in novel habitats, and causing restricted gene flow for those in the original environment, leading to evolutionary divergence.

1.3 Marine larvae and phenotypic plasticity

The majority of marine organisms produce planktotrophic larvae (Thorson, 1950; Dupont, Dorey and Thorndyke, 2010). Strathmann (1993) reviews various hypothesis for the origins of larval life history stages, showing that although larval development is in at least 15 marine phyla, the origins of larval development vary inter and intra-phylogenetically. He suggested that feeding methods and locomotion are the distinct characteristics that distinguish between planktonic larvae and benthic postlarval/juvenile stages (Strathmann, 1993). Both feeding and locomotion are seen to influence larval form, with dramatic changes in larval form originating from a loss or change of either of these structures, especially for those that accompany the loss of larval feeding, as well as complete loss of larval stages (Strathmann, 1993). Larvae have been shown to possess basic chemoreception (Arvedlund and Kavanagh, 2009; Chase, Dijkstra and Harris, 2016), sound detection (Leis, Carson-Ewart and Cato, 2002), and photodetection (Villamizar *et al.*, 2011), which is indicative of their complexity. Furthermore, many marine species during their larval development exhibit complex changes through metamorphosis which lead to different morphologies and physiologies, resulting in different traits and behaviours suited to their environment (Strathmann, 1993).

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As previously stated, it is likely that plasticity derives from environmental heterogeny (see section 1.2); however, additional drivers, such as those related to climate change are likely to elicit a passive plastic response (Whitman and Agrawal, 2009) (section 1.1 Introduction). Understanding how marine larvae express passive plastic responses when subject to projected climate change provides information into how future ecological communities may be affected (Gaines and Roughgarden, 1985; Hoegh-Guldberg and Pearse, 1995; Van Buskirk, 2002; Byrne and Przeslawski, 2013).

Oliphant, Hauton and Thatje (2013) studied the effects of temperature (15, 20, and 25 °C) on the larval development of *Palaemonetes varians*. Increased temperature led to faster development, with those developed at 25 °C passing through fewer instar stages within their development. Having fewer instars during larval development suggests that larvae skipped stages, and therefore radical metamorphosis could have meant an additional energetic cost to the larvae, as increased development rate for an organism to 'skip' instars would mean that the organism is producing a morphology that is more different in a reduced amount of time. This may be the reason for the resulting smaller size for larvae developed at 25 °C. Developmental plasticity during early development has also been shown to impact the development of adult life stages and organismal fitness (Gaines and Roughgarden, 1985).

Accelerated development in response to elevated temperature aligns with predictions of Arrhenius theory, which relates to Arrhenius kinetics whereby a 10 °C increase in temperature will double the rate of a biochemical reaction (Arrhenius, 1915; Knies and Kingsolver, 2010; Mundim *et al.*, 2020). When related to physiology, Arrhenius kinetics only relate to a narrow temperature range in cold blooded organisms due to biological rate limitations (Knies and Kingsolver, 2010), but the Q_{10} theory, related to

Arrhenius kinetics, is inclusive of the limits and do not always conform with the predictions based on Arrhenius law (Mundim *et al.*, 2020). This non-conformance is usually related to the limits of the Q_{10} theory, where biological processes plateau and decline due to the denaturing of proteins and enzymes when subject to less-than optimal temperature (Knies and Kingsolver, 2010; Mundim *et al.*, 2020). One study (Quinn *et al.*, 2013) investigates the larvae *of Homarus americanus* (the American lobster) originating from colder waters, which expressed slower development in response to elevated temperatures. Furthermore, Quinn *et al.* (2013) have shown that their warm water counterparts do express increased development rates when subject to warmer temperatures, suggesting that there is geographic variation to the plasticity that larvae can express and rejecting the temperature coefficient as a universal model.

Other widely recognised anthropogenic factors is increased CO_2 in the atmosphere, which can diffuse into the ocean, reducing pH levels (Doney *et al.*, 2009; Breitburg *et al.*, 2015). Cole and colleagues (2016) show that larvae of *Ostrea angasi* raised in increased CO_2 have a 3 % reduced body size, with no effect on mortality or morphology. Larvae exposed to increased temperature and lower salinity had a significantly higher mortality rate, regardless of exposure to increased CO_2; however, neither increased temperature nor salinity alone showed this effect. When CO_2 is studied under the additional stress of increased temperature or salinity, *Ostrea angasi* larvae present far more morphological abnormalities (Cole *et al.*, 2016). This study shows how multiple drivers can interact to express varying degrees of effect to an organism. Typically, marine larvae are an especially vulnerable life history stage (Cowan, Houde and Rose, 1996; Dupont, Dorey and Thorndyke, 2010) and many pelagic larvae lack active motility and are therefore unable to counter the currents of the ocean (De

Wolf, 1973). Most larvae will be subject to predation from planktivorous predators making response to predation threat particularly important in these groups.

The abundance of marine larvae as a form of life history development is likely due to their various developmental modes, including pelagic development that can detect complex changes in the environment from very early on and respond to this with complex phenotypically plastic traits that increases the chance of survival (Hoegh-Guldberg and Pearse, 1995; Przeslawski, Byrne and Mellin, 2015). This life history stage gives insight into ecological function and communities under future projected climate conditions (Byrne and Przeslawski, 2013); however, these plastic traits are not only related to abiotic drivers, as marine larvae have shown to express plasticity from the induction of biotic drivers (Hoegh-Guldberg and Pearse, 1995; Hansson, 2004; Coors and Meester, 2008; Vaughn and Strathmann, 2008; Charpentier, Wright and Cohen, 2017). These biotic drivers can be inclusive of predator exposure, which results in the phenotypically plastic response of included defences (Magnhagen and Forsgren, 1991; Vaughn and Strathmann, 2008; Charpentier, Wright and Cohen, 2017; Touchon and Robertson, 2018).

1.4 Induced defences in marine larvae

Predation stress can initiate the expression of induced defences, a form of phenotypic plasticity that increases the chance of survival under predation threat (Tollrian and Harvell, 1999; Van Donk, Ianora and Vos, 2011). These responses are likely to be an example of active plasticity, due to the complexities of the responses involved for those expressing induced defences (Whitman and Agrawal, 2009). Induced defence responses may be chemical, behavioural or morphological with many examples having been described across a range of phyla (Tollrian and Harvell, 1999). Morphological

structures presented by prey due to predator exposure may be adaptive or maladaptive to prey survival, with the evolution of a temporary, inducible, structure being favoured when the defence incurs a cost to fitness or when predation risk fluctuates (Brönmark and Miner, 1992). Induced defences are presented in an array of organisms, including plants and phytoplankton that express chemical and morphological defences to deter being eaten (Van Donk, Ianora and Vos, 2011). Induced defences have also been extensively studied in aquatic organisms, such as anuran larvae, Crucian carp, periwinkles and *Daphnia sp*. (Brönmark and Miner, 1992; Laurila *et al.*, 2002; Laforsch, Beccara and Tollrian, 2006; Bibby *et al.*, 2007).

Marine larvae express a wide variety of induced defences, including altered shell morphology (Vaughn, 2007), cloning *via* budding (Vaughn and Strathmann, 2008) and changes in hatch timing (Miner, Donovan and Andrews, 2010). *Dendraster excentricus* larvae have shown to clone themselves *via* budding under predation threat (Vaughn and Strathmann, 2008; Vaughn, 2010). Budding is the formation of a clone in which the larvae splits, or buds, whilst also reducing its growth rate. Smaller body size suggests an advantage when avoiding visual predators, predators are more likely to select larger prey when actively hunting and small size may avoid getting caught in the gill rakers of filter feeders.

Vaughn (2007) also shows that gastropod larvae are capable of morphological induced defences to aid predator avoidance from *Cancer spp.* zoea larvae. Here, it is shown that *Littorina scutulata* larvae are capable of expressing a smaller operculum and rounder shells that reduces predation by inhibiting shell access for crab zoea larvae, allowing more room for *L. scutulata* to retract further in the shell as well as forming a shell shape that is harder to crush (Vaughn, 2007; Solas *et al.*, 2015). Crab zoea have

shown to express induced defences when subject to fish kairomones (Charpentier and Cohen, 2015; Charpentier, Wright and Cohen, 2017). Kairomones are 'secondary metabolites' produced as waste during metabolism, that induce an adaptively favourable physiological or behavioural response for the receiver, but not the emitter (Brown, Eisner and Whittaker, 1970; Kost, 2008). Therefore, the use of kairomones allows for predation pressure in experiments to be simulated chemically, making it a method with significant advantages over the use of visual or physical cues to induce predator responses (Forward and Rittschof, 1999; Bhat, Greulich and Martins, 2015; Mezrai *et al.*, 2020). When crab zoea of both *Rhithropanopeus harrisii* and *Hemigrapsus sanguineus* are subject to fish kairomones, the larvae express heightened visual sensitivity which aids predator avoidance, as well as spine elongation to reduce predation (Charpentier and Cohen, 2015; Charpentier, Wright and Cohen, 2017). Charpentier, Wright and Cohen (2017) also note the fact that larvae from smaller broods are more capable of expressing increased spine length, stating that this is likely linked to their larger larval size.

Marine larvae are capable of complex phenotypically plastic responses to environmental drivers (section 1.3 Marine larvae and phenotypic plasticity) as well as predator induced defences (Vaughn, 2007; Przeslawski, Byrne and Mellin, 2015; Touchon and Robertson, 2018). However, the study of marine larval responses to multiple drivers remains in its infancy (Przeslawski, Byrne and Mellin, 2015), and further research will help elucidate developmental plasticity during this stage, and carry-over effects resulting from such responses. (Hoegh-Guldberg and Pearse, 1995; Simith, Diele and Abrunhosa, 2013; Przeslawski, Byrne and Mellin, 2015; Cole *et al.*, 2016; Parker *et al.*, 2017; Moore and Martin, 2019).

1.5 Plasticity and multiple drivers

A surge of interest for investigating phenotypic plasticity in multiple drivers has occurred in recent years due to the increased potential for ecological realism from multifactorial studies compared to single driver studies (Kraufvelin, 1999; Przeslawski, Byrne and Mellin, 2015). Ecological realism is a contentious idea, because for 'true' ecological realism, thousands of environmental drivers would need to be incorporated to fluctuating simulate conditions *in situ* (Letellier and Aziz-Alaoui, 2002). For the sake of experimental laboratory research, ecological realism, the term suggested by Kraufvelin (1999), is the similarity between the artificial systems and *in situ* environments. Multifactorial designs are not always representative of *in situ* conditions, and the drivers need to be used in the correct sense (Kraufvelin, 1999; Przeslawski, Byrne and Mellin, 2015). For example, investigating one driver, e.g. temperature, and ensuring it mimics *in situ* conditions, as well as those *in situ* conditions in a projected climate, may provide more ecologically relevance than those investigating multiple static drivers, some of which the organism may not experience (Kraufvelin, 1999; Przeslawski, Byrne and Mellin, 2015). This interest has likely been driven by the multiple anthropogenic drivers that organisms are increasingly experiencing *in situ*, and a need to further understand the interactions and sensitivities of these drivers (Przeslawski, Byrne and Mellin, 2015). If multiple drivers are not taken into consideration, the effects of those drivers on organism response will be unpredictable due to the various interactions they may have (Daufresne and Boët, 2007).

Synergistic responses are those where the interactive response of a phenotypically plastic trait subject to two drivers is much larger than the response to those drivers individually (Folt *et al.*, 1999). Tollrian and Laforsch (2006) investigate the

known response of *Daphnia cucullata* to both predation and turbulence, both of which increase helmet size of *D. cucullata* individually. The interactive effect of these drivers results in a synergistic response where helmets reached maximum size found in nature, which was significantly larger than induced by these drivers in isolation. Przeslawski, Byrne and Mellin (2015) state that additive responses are those where the phenotypically plastic response to two environmental drivers is equal to the sum of those drivers individually, whereas antagonistic responses are when the interactive response is less than the sum of those induced by drivers in isolation.

Folt et al (1999) also discuss additive response to multiple drivers, using this as a baseline to discuss synergistic and antagonistic plastic responses. Additive responses can be seen in *Littorina littorea* when subject to ocean acidification and increased temperature (Melatunan et al., 2013). Separately, these two drivers reduce shell weight of adult *L. littorea*, but the interaction of these two drivers further reduces shell weight equal to that of the effect of these two drivers combined individually. *Chaoborus crystallinus* larvae predation and pesticide exposure on *Daphnia magna* results in an antagonistic response (Coors and Meester, 2008), where those exposed to these drivers individually had a significant reduction in the amount of living first-brood offspring produced, but the interaction of these two drivers reverted the amount of living first-brood offspring to similar quantities of individuals kept in control conditions.

It has been suggested that acclimation to drivers may alter multiple driver interactions (Collins *et al.*, 2021). In this review, Collins *et al* (2021) look at the interaction of temperature and hypoxia, and how acclimating organisms to increased temperature may alter phenotypically plastic responses to hypoxia compared to subjecting the organisms to acute thermal increase and hypoxia. With acute

temperature increase lessening the hypoxic response for organisms, it was suggested that organisms acclimated to increased temperature may possess the ability for improved hypoxic function; however, examples of this were only found in fish, no evidence was found among crustacea, and very limited examples for molluscs (Collins *et al.*, 2021). Acclimating organisms to certain drivers may represent how those organisms acclimate to projected climate change conditions by 2100, providing a deeper understanding of the interactive effect of multiple drivers on developing organisms (Suckling *et al.*, 2015; Havird *et al.*, 2020; Collins *et al.*, 2021).

Przeslawski, Byrne and Mellin (2015) state that a deeper understanding of physiology is likely to explain the complex responses organisms express when subject to multiple drivers, as this is likely to help increase the precision of predictions for organisms in the same taxonomic groups, as those are most likely to express a similar response. It should also be noted that although multiple driver studies may present more ecologic relevance compared to some single driver studies, they still over simplify the complexity of the natural habitat (Przeslawski, Byrne and Mellin, 2015).

Multiple drivers are unlikely to be solely abiotic, although many drivers immediately considered in relation to climate change are abiotic, for example elevated temperature, ocean acidification and hypoxia (Harley *et al.*, 2006; Doney *et al.*, 2012; Cole *et al.*, 2016). Not only is climate change inclusive of abiotic factors, but biotic factors as well, such as food availability and invasive species. (Berglund and Bengtsson, 1981; Claramunt and Wahl, 2000; Przeslawski, Byrne and Mellin, 2015). The review, from Przeslawski, Byrne and Mellin (2015), shows that marine embryo and larval response to the interactions of multiple drivers are most commonly synergistic, followed by additive and antagonistic simultaneously, and that investigating multifactorial designs allows for

a degree of ecological realism, as mentioned previously (section 1.5 Plasticity and multiple drivers) (Przeslawski, Byrne and Mellin, 2015; Gunderson, Armstrong and Stillman, 2016). Investigating multiple stressors allows insight into how these factors may interact with one another. This does not only apply to abiotic factors, although this is what has had more substantial investigation, but this also goes for abiotic and biotic factors, with few studies investigating the interactions of environmental drivers such as predation threat, or food availability, simultaneously with an abiotic factor.

Claramunt and Wahl (2000) investigate the interaction of abiotic and biotic drivers in the field. Here, they selected various reservoirs in which fish larvae were located and summarised the abiotic and biotic factors that these larvae were subject to. Growth rate was then measured by investigating the daily rings in the otoliths of individuals to show how these factors interacted with one another. One thing that was noted is that abiotic drivers tended to influence fish larvae in a more general sense, whereas biotic drivers, such as larval density and food availability, tended to be more specific to the species. This study gave a good insight into the complex interactions of abiotic and biotic drivers. Being able to replicate a complex study such as this in the lab would be difficult, due to natural fluctuations of factors found in nature (Przeslawski, Byrne and Mellin, 2015)

Adult littorinid snails, such as *Littorina obtusata*, exposed to effluents of *Carcinus maenas* feeding on conspecific snails develop thicker shells, which likely reduces the efficiency of predator crushing ability (Trussell, 1996; Trussell and Nicklin, 2002; Brookes and Rochette, 2007). This has also been represented in *Littorina littorea*, with Bibby and colleagues (2007) exposing the periwinkles to ocean acidification to study the interaction of abiotic and biotic drivers on the shell thickness of adult *L. littorea.* Those

in the presence of predation expressed the thicker shells, just as *L. obtusata* had in the aforementioned studies; however, when subject to a lower seawater pH, *L. littorea* experienced hypometabolism and were unable to produce thicker shells. To compensate for this, the periwinkles did express noted behavioural changes that would increase predator avoidance (Bibby *et al.*, 2007).

Dixon, Munday and Jones (2010) investigate how ocean acidification influences predator avoidance through disruption in chemoreception for anemonefish larvae, *Amphiprion percula*, causing them to have a strong attraction to predator cues under the exposure of a lowered pH. This has also been shown to influence the predators, who have an inability to detect their prey when subject to lowered pH (Cripps, Munday and McCormick, 2011), resulting in the predator avoiding the prey odour. This study uses the same fish, *Pseudochromis fuscus*, as the aforementioned study that investigates lowered pH on predator avoidance in anemonefish larvae. Due to the effects of ocean acidification on both predator and prey (Munday *et al.*, 2009; Dixson, Munday and Jones, 2010; Cripps, Munday and McCormick, 2011), it is likely that the interaction between the two will be distorted by exposure to ocean acidification and altering their relationship.

1.6 Thesis aims and objectives

The main aim of this thesis was to investigate how predation threat (fish kairomones) and thermal drivers (i.e. increased temperature) affected the development and post-hatching performance of *Littorina littorea* embryos and larvae. This was achieved by: 1) Exposing developing *L. littorea* embryos to a factorial design of two temperatures mimicking *in situ* (15 °C) and those projected by 2100 (20 °C), and the presence or absence of predator kairomones (fish mucus) and measuring: i) the

development time and morphology of embryos; ii) carry over effects on the performance (swimming) of hatchling larvae, including how performance was affected under acute exposure to predator kairomones.

Chapter Two: The combined effects of temperature and predation stress on early life stages of the marine gastropod Littorina littorea

2.1 Introduction

Phenotypic plasticity is the ability for an organism to express multiple phenotypes from one genotype under environmental stress (Agrawal, 2001; Whitman and Agrawal, 2009). This ability is predicted to be most pronounced in species that experience a range of environmental conditions (Auld, Agrawal and Relyea, 2010; Pfennig *et al.*, 2010) and to play a role in enabling species to adapt to rapid environmental change (West-Eberhard, 2005; Pfennig *et al.*, 2010; Dayan, Crawford and Oleksiak, 2015). Whilst initial investigations of plasticity tended to focus on responses to single environmental factors in isolation, there has been more recent interest in investigating how multiple factors might initiate plastic responses (Coors and Meester, 2008; Gunderson, Armstrong and Stillman, 2016). Indeed , it has been argued that investigating multiple drivers allows for a better understanding of ecological realism (see section 1.5 Plasticity and multiple drivers) described by Kraufvelin (1999) as the “degree of similarity between the artificial system and the natural ecosystem”.

Studies that investigate multiple drivers have increased rapidly in recent years, many looking at multiple drivers associated with projected climate change (Harley *et al.*, 2006; Byrne and Przeslawski, 2013; Przeslawski, Byrne and Mellin, 2015; Cole *et al.*, 2016; Gunderson, Armstrong and Stillman, 2016; Kingsolver and Buckley, 2017). Interactions between environmental drivers can be complex (Weinig, 2000), and it is theorised that

organisms that express a phenotypic trait in one environment may limit the plastic response in another environment (Auld, Agrawal and Relyea, 2010). This is described as an 'intrinsic genetic cost' by van Kleunen and Fischer (2005) (see section 1.2 Phenotypic Plasticity). For example, echinoderms have been shown to express an antagonistic response to temperature increase and ocean acidification, where the interaction of the two drivers is less than the sum of those drivers separately, when subject to multiple stressors (Brennand *et al.,* 2010). Most multiple driver studies investigate the interaction of abiotic drivers, a limited number look into the interactions of abiotic and biotic drivers (Hoegh-Guldberg and Pearse, 1995; Claramunt and Wahl, 2000; Coors and Meester, 2008; Przeslawski, Byrne and Mellin, 2015; Cole *et al.*, 2016; Gunderson, Armstrong and Stillman, 2016), which allows for a more ecologically realistic insight into future climate conditions as projected climate change is unlikely to solely affect abiotic stressors (see section 1.5 Plasticity and multiple drivers) (Kraufvelin, 1999; Przeslawski, Byrne and Mellin, 2015; Gunderson, Armstrong and Stillman, 2016).

Environmental conditions in marine systems are often highly variable and these habitats are also susceptible to anthropogenic environmental change. Hence, they are appropriate systems for studying the effects of multiple environmental drivers on plastic responses. Phenotypic plasticity has been extensively studied in many marine organisms (e.g. Pfennig *et al.*, 2010; Padilla and Savedo, 2013; Przeslawski, Byrne and Mellin, 2015; Kingsolver and Buckley, 2017), with a range of responses, including alterations in behaviour, the development of morphological traits, and chemoreception (Magnhagen and Forsgren, 1991; Hansson, 2004; Vaughn, 2007, 2010; Munday *et al.*, 2009; Mery and Burns, 2010; Przeslawski, Byrne and Mellin, 2015; Chan *et al.*, 2016; Charpentier, Wright and Cohen, 2017). Marine ecosystems have also been used as model systems for the

study of biotic interactions (Berglund and Bengtsson, 1981; Hoegh-Guldberg and Pearse, 1995; Claramunt and Wahl, 2000; Vaughn, 2010; Charpentier, Wright and Cohen, 2017) and, more recently the interaction between such biotic interactions and abiotic stressors (Berglund and Bengtsson, 1981; Claramunt and Wahl, 2000). For example, those that look at how ocean acidification affects the induced predator response (Bibby *et al.*, 2007; Munday *et al.*, 2009; Dixson, Munday and Jones, 2010), show those exposed to ocean acidification express poor predatory avoidance behaviours. Very few studies investigate the interactions between predation threat and temperature increase (Allan *et al.*, 2015; Mira-Mendes *et al.*, 2019). Temperature increase has been shown to cause an increase in metabolic rate and development, as well as changes in morphology (Robinson, Peters and Zimmermann, 1983; Melatunan *et al.*, 2013; Pan and Herbing, 2017; Ruthsatz *et al.*, 2018), whereas predation has been shown to influence morphology and behaviour (Magnhagen and Forsgren, 1991; Hansson, 2004; Vaughn, 2007); therefore, the combination of both predation threat and temperature increase may have an interactive effect due to overlapping affected traits.

A large majority of marine organisms produce planktotrophic larvae (Thorson, 1950; Dupont, Dorey and Thorndyke, 2010), one of the most vulnerable life history stages for an organism (Cowan, Houde and Rose, 1996; Dupont, Dorey and Thorndyke, 2010) and their survival is the foundation for ecological communities (Gaines and Roughgarden, 1985; Przeslawski, Byrne and Mellin, 2015). A recent focus on plasticity in marine larvae has provided varying examples (Clegg *et al.*, 2000; Oliphant, Hauton and Thatje, 2013; Ruthsatz *et al.*, 2018), such as cloning *via* budding (Vaughn and Strathmann, 2008; Vaughn, 2010) and morphological plasticity.

The overall aim of this study was to investigate the combined effect of increased temperature (abiotic factor) and predator kairomones (biotic factor) on developing embryos and larvae of the marine gastropod *Littorina littorea*. Specifically, developing embryos were subject to a 5 °C increase in temperature, taking the temperature 1 - 4 °C above the yearly mean highest temperature of between 16 - 17 °C to 20 °C, which is within climate change projections for 2100 (Cooper, 1958; Laffoley and Baxter, 2016). Also investigating how increased temperature affected their induced defence response (embryonic development and larval swimming performance) when exposed to predatory fish kairomones. The objectives of the study were: 1) to assess the effects of temperature and predator kairomones on the development time and size of *L. littorea* embryos; 2) to compare the effects of developmental exposure to increased temperature and predator kairomones on the performance (swimming behaviour) of hatched veliger larvae; 3) to investigate effects of acute vs chronic exposure to predator kairomones on swimming behaviour and performance of hatched veliger larvae. It was hypothesised that predation threat and temperature would interact with their effect on the development and morphology of *L. littorea*, and result in carry-over effects on the performance of hatchling larvae.

2.1.1 Study species

Littorina littorea (Lineaus, 1758) (Fig 2.1), or the common periwinkle is found in lower intertidal zones of rocky shores. Here, it resides in rock pools at low tides and is frequently subject to harsh conditions (Saier, 2000; Tomanek and Helmuth, 2002). Adult *L. littorea* have been shown to be phenotypically plastic when subject to predation, ocean acidification and temperature (Bibby *et al.*, 2007; Melatunan *et al.*, 2011), with

responses including thicker shells, thinner shells and reduced respiration rate, respectively.

Male *L. littorea* internally fertilise females (Robson and Williams, 1971) during the breeding season, which typically runs from January through to June (Fish, 1972). Each female releases hundreds of eggs, which are approximately 0.14 mm in diameter, into the water column daily (Fish, 1972; Grahame, 1975). These eggs develop into pelagic larvae within a few days, but remain in the water column for up to 7 weeks (Fish, 1972; Grahame, 1973). Adults regularly produce fertilised eggs throughout the breeding season, which allowed for a large abundance of embryos to be collected on a regular basis.

Figure 2.1. Image of *Littorina littorea.* Scale Bar = 5 mm

Source: WoRMS - World Register of Marine Species, Author - Claude Nozères

L. littorea were used as a model organism for this study due to their ease of laboratory handling, reproductive output, their well-documented larval and adult life history stages, as well as the previous work on predator responses (Newell, 1958; Robson and Williams, 1971; Grahame, 1973; Kemp and Bertness, 1984; Saier, 2000; Bibby *et al.*, 2007). Despite increasing evidence of complex responses of early life stage

marine organisms to combined environmental drivers (Vaughn and Strathmann, 2008; Charpentier and Cohen, 2015; Przeslawski, Byrne and Mellin, 2015), there remain significant gaps in our understanding. This is particularly true when considering not just abiotic environmental drivers, but also their interaction with biotic drivers.

2.2 Methods

2.2.1 Organism Culture

Adult *Littorina littorea* (n = 120) (size > 1.2 cm) were collected from the intertidal zone at Cawsands beach, UK (50.331058, -4.201766) in May 2019. Cawsands beach is an east-facing shingle beach with rocky intertidal areas that resides within the Plymouth sound (Johnson, 1890). Once adult *L. littorea* were collected, they were transported to the laboratory. They were acclimated for 24 hours to laboratory conditions in 20 l aquaria containing 12 l of aerated UV filtered seawater (UVFSW) (temperature = 15 °C, salinity = 35 ppt, pH 8.2, 12 h light: 12 h dark cycle). *Ulva lactuca* fronds collected at the same site were soaked in reverse osmosis (RO) water for 10 minutes before being fed *ad libitum* to the snails. Full water changes were conducted every 3 days, and any remaining *U. lactuca* was removed and replaced. Newly-laid egg capsules, containing embryos, were collected every 24 hours from the adult population by filtering aquarium water through a 400 µm mesh net.

2.2.2 Experimental protocol

2.2.2.1 Experimental design

Embryo and larval exposure to predator presence and increased temperatures were applied using a two-factor design to allow investigation of potential interactive effects of these two factors as well as their effect in isolation. A 5 °C temperature

increase from 15 °C to 20 °C takes the larvae from a temperature the larvae and adults will be regularly subject to subject to (15 °C) and taking the temperature 1 - 4 °C above the yearly mean highest temperature of between 16 - 17 °C to 20 °C, which is within climate change projections for 2100 (Cooper, 1958; Laffoley and Baxter, 2016). Embryos were collected within one day, which took approximately three hours to obtain 120 embryos that had reached the 8-cell stage. These were then transplanted to individual wells of two 96-well multiwell plates (Nunc™, Microwell™) containing 300 µl FSW (salinity = 34 ppt, pH = 8.25 under 12 h light: 12 h dark cycle; plates maintained at either 15 °C or 20 °C). Of the 60 embryos in each plate, half were subject to chronic predation exposure, where they received an inoculation of predator mucus (0.05 mg wet mass mucus per ml FSW) (see below for preparation). Developing *L. littorea* were maintained at their respective temperatures in constant temperature (CT) rooms, and multiwell plates were sealed using Aeraseal (Excel Scientific, Inc., USA) and covered with an upside-down 3 l plastic aquarium to reduce airflow directly over the plates, while still sustaining adequate oxygen diffusion (Appendix 1). Embryos received 90 % water changes every other day *via* manual pipetting, and those in the chronic predator presence treatment cues received fresh mucus at this time.

Embryos were observed every 24 h to check for mortality and deformity, as well as collection of hatched larvae. This meant that within 24 h of hatching (where larvae were free-swimming outside of the egg), larvae were transferred to a 96 multiwell plate containing 300 µl FSW and maintained at the same temperature subject to during development. Plates were sealed with Aeraseal, covered with an upside-down 3 l plastic aquarium to reduce airflow, and left for 24 h.

Following this, each larva was transferred to a separate well of a 96 multiwell plate, which contained 150 µl FSW sustained at their respective temperature of either 15 °C or 20 °C. Videos of larvae were taken using procedures described below. Following video acquisition, the larva was transferred, using a 100 – 1000 µl micropipette, to a separate well of a 96 multiwell plate containing 150 µl 0.05 mg ml^{-1} (wet mass) fish mucus for acute exposure to predator kairomones, and sustained at their reciprocal temperature of 15 °C or 20 °C for a period of 1 h before further videos could be taken using the same procedures described below. This allowed for investigation of the post-hatch behavioural response to predator exposure, and whether there was a carry-over effect from chronic developmental exposure (Simith, Diele and Abrunhosa, 2013; Ituarte *et al.*, 2014; Przeslawski, Byrne and Mellin, 2015).

2.2.2.2 Preparation of predation cue

Mucus from a range of planktivorous fish species was used as a way of obtaining a standardised and generalised predator presence cue (Walsh *et al.*, 2015). This mucus mixture was obtained from four planktivorous species found off of the south west coast: *Clupea harengus*, *Scomber scombrus*, *Sardina pilchardus* (Checkley, 1982; Nikolioudakis *et al.*, 2012; Bachiller *et al.*, 2016), all of which are planktivorous their entire lives and *Dicentrarchus labrax*, which are planktivorous as juveniles (Cahu and Infante, 1994). As these fish are common in the Plymouth sound and surrounding areas, *L. littorea* embryos and larvae would be regularly exposed to them in the ocean.

C. harengus, *S. scombrus*, *S. pilchardus* and *D. labrax* were collected from local fishmongers, where they had been caught same day. From here, they were taken to the lab where they were placed into refrigeration for approximately 30 minutes, until mucus was collected same day. Mucus was collected using the method developed by Vaughn

(2010). Preweighed KIMTECH Science© Precision wipes (Kimberly-Clark Worldwide, Inc.), cut to 1 inch square sheets, were used to blot the surface of individual fish, avoiding the anal fin and surrounding areas to reduce risk of contamination from internal body fluids (Stabell and Selset, 1980). Mucus-soaked Kimwipes were weighed, and quantity of wet-mass mucus was calculated by the increase in weight of Kimwipes. Mucus-Kimwipes were placed in an appropriate quantity of 0.45 µm filtered sea water (FSW) (salinity = 34 ppt, pH = 8.25, temperature = 15 °C) to a concentration of 10 mg ml^{-1} FSW (wet mass) fish mucus where the wipes were left for an hour, being frequently stirred. The resulting solution was then placed into individual 50 ml Falcon conical centrifuge tubes and frozen for a maximum period of 14 days prior for use in the study (Vaughn, 2010). Fish mucus samples were subsequently placed into a refrigerator and allowed to defrost overnight as needed during the course of the study. Fish mucus samples from each fish species was combined equally to a total concentration of 0.05 mg ml^{-1} (wet mass) fish mucus, using FSW stored at either 15 °C or 20 °C to dilute.

2.2.2.3 Image acquisition

Each larva was recorded for 60 s at 17 frames per second at 7.8x magnification using a QImaging R6 Retiga camera operating at 2 MP resolution (QImaging, Surrey, Canada) mounted on a microscope (Leica M205 C, Leica Microsystems, Wetzlar, Germany) and controlled using MicroManager (version 1.4, Edelstein *et al.*, 2014). Following this initial period of image acquisition, the larva was exposed to 0.05 mg ml^{-1} (wet mass) fish mucus for an hour (hereafter after referred to as acute exposure), and another 60 s video was captured using the same procedures as before.

During behaviour video analysis, it was noted whether each larva was moving or stationary. To measure larval morphology the water volume was reduced (using a 20-

200 µl micropipette) to approx. 50 µl to reduce mobility, and larvae were imaged at 160 x magnification.

2.2.2.4 Data acquisition

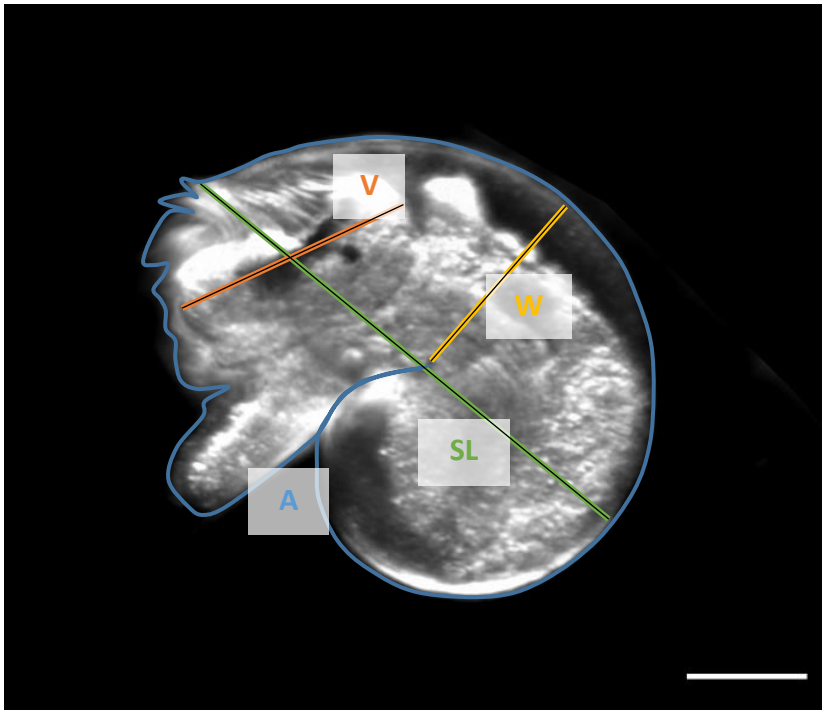

Figure 2.2 *Littorina littorea* larva 24 hours post-hatch with morphological measurement labels. V = Velum; W = Width of First Whorl at intersection; SL = Shell Length; A = Area. Scale bar = 500 µm

Development was measured as time in days from embryo collection, approximately 24 hours after being laid. For each 24-hour post-hatch larva, the shell length, velum length, width of first whorl at intersection (hereafter referred to as width) and whole-body area (hereafter referred to as area) were measured (Fig. 2.2), using a stage micrometer to standardise measurements. Velum length was used as a proxy for velum area (Appendix 2).

Larval movement was quantified from the videos of each larva swimming at 17 frames per second using the ImageJ (Schneider, Rasband and Eliceiri, 2012) plug-in TrackMate (Tinevez *et al.*, 2017). TrackMate tracks individuals from frame to frame (Fig. 2.3) and was calibrated to track the movement of individual larva automatically after

telling the software which moving object is the larva and calibrating the settings to identify its specific intensity and size. Inconsistencies in lighting and multiple point detection were mitigated via frames being individually checked to ensure that the larva was being tracked consistently. TrackMate takes information on the X and Y location of the particle for each frame of the video, and then tracks the distance travelled between each frame, with a value of the average distance travelled between each frame for the entirety of the video. Other data collected during video acquisition includes the total displacement of the particle, which is the distance travelled during the video between the start and end point.

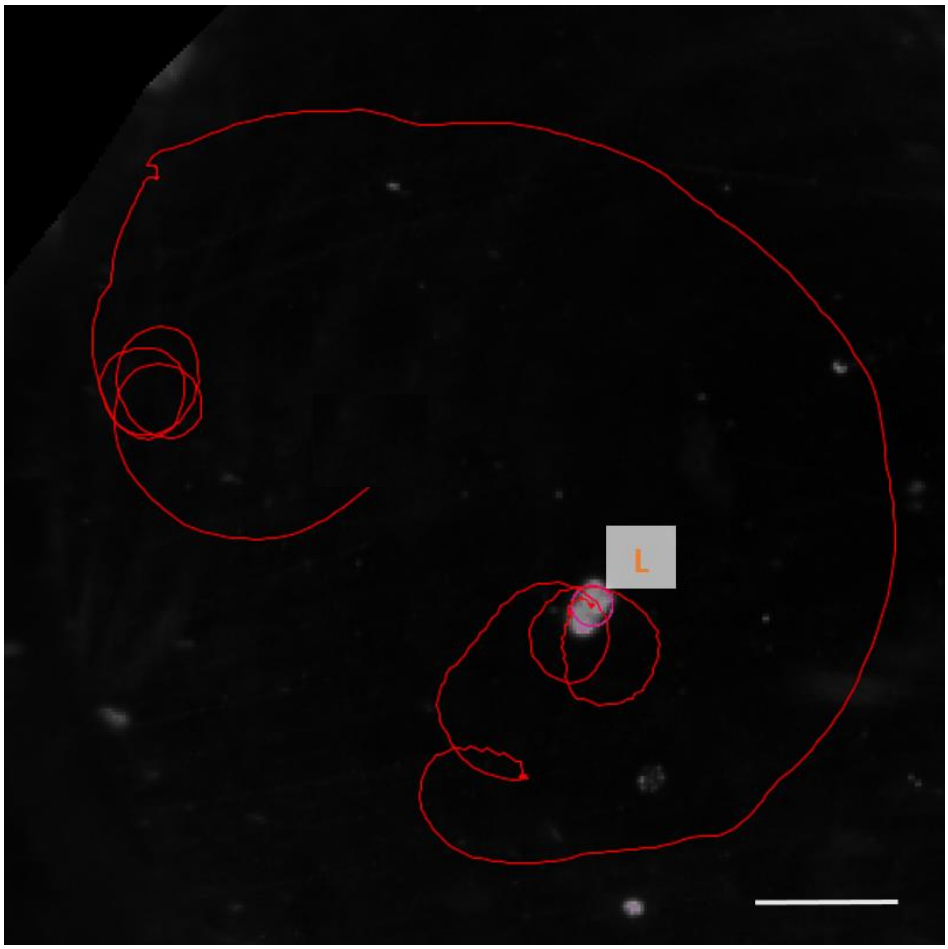

Figure 2.3 TrackMate analysis of image sequences, tracking the larva movement (L) in red. Scale bar = 5mm

2.2.3 Data Analysis

The effects of temperature, predator kairomones and their interaction on hatching time were analysed using a general linear model (GLM). Analysis was attempted using a PERMANOVA but resulted in similar results that needed further division to analyse data efficiently, and running a GLM provided the possibility for separation of results that insignificantly interacted. Hatching time data were positively skewed and could not be transformed for normal distribution; therefore, data were analysed using the Poisson error family which allows for the positive skew. Data were largely over-dispersed, therefore requiring analysis using the Quasi-Poisson error family, which accounted for over-dispersion.

Some larvae never moved during the entire video, and movement was categorised as either 1 or 0, depending on whether the larvae were moving or stationary, respectively, whilst the video was taken. This resulted in binary data that could be analysed using a general linear model (GLM). The binary data set was not normally distributed and best analysed using the binomial error family, which allowed for an analysis of the proportion of moving:stationary larvae across treatments, investigating the interaction of temperature and predator kairomones for larval movement. Mean movement speeds were acquired from TrackMate (Tinevez *et al.*, 2017) and analysed with a general linear model (GLM) to see whether larvae subject to predator kairomones and/or increased temperature during development had differing swimming speeds than those subject to predator absence at 15 °C (control). The analysis also covers hour exposure to see whether this has an effect on the swimming behaviour of 24 hour post-hatch larvae, whilst also investigating the covariate strength of the velum size on movement speeds, as the velum is the means of locomotion. These data were not

normally distributed, representing a gamma distribution. This required the data to be analysed using the gamma error family in the GLM (Appendix 3).

Due to the significant result from the GLM, regarding correlation between the velum size and movement speeds, the data were separated into those developed under predator presence and absence, and the correlation was measured using a Pearson's r correlation test for each data set.

2.3 Results

2.2.1 Development time

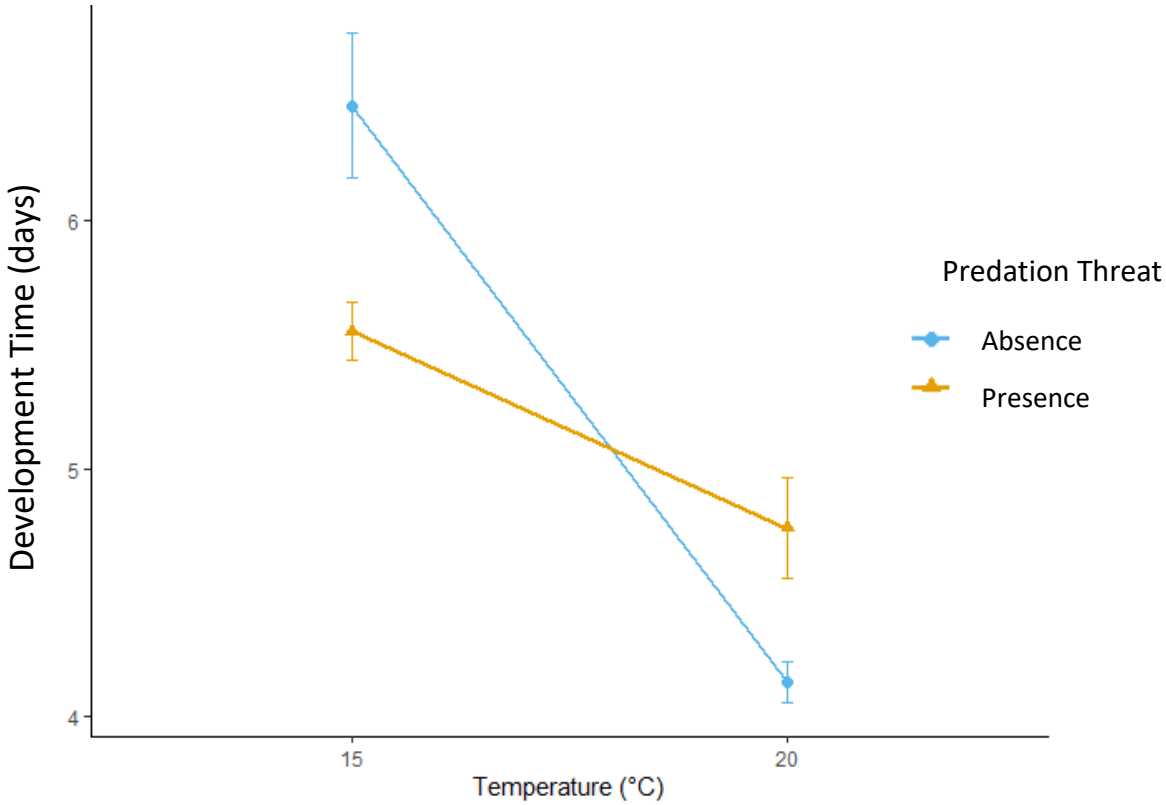

Figure 2.4 Development time (days) of *Littorina littorea* larvae at 15 °C and 20 °C and in the presence or absence of predator kairomones. Mean ± 1 S.E.

There was a significant interactive effect of temperature and predator kairomones on development time (linear regression, $\chi = 2.9443$, $P < 0.001$) (Fig. 2.4). At 15 °C, predator kairomones significantly lengthened the development time by 14 %, whereas it was 15 % shorter at 20 °C for those exposed to predator kairomones. Although the

interaction between the two drivers could not be separated due to the generalised linear model used, an analysis of means using raw data show that development time under absence of predation at 20 °C was 36 % shorter than those developed at 15 °C. Those exposed to predator kairomones at 20 °C had a 15 % shorter development time compared to those developed at in the control treatment.

2.3.2 Hatchling morphology

Shell length for hatchlings was 20 % larger in the presence of predator kairomones (Fig. 2.5. A) (ANOVA, $F_{1, 76} = 69.355$, $P < 0.001$), whereas width was 10 % smaller in the presence of predator kairomones (ANOVA, $F_{1, 76} = 21.7012$, $P < 0.001$). Velum size for hatchlings developmentally exposed to predator kairomones was 22 % larger compared to those developed in predator absence (ANOVA, $F_{1, 66} = 41.0001$, $P < 0.001$) and the total area of hatchlings was also found to be 21 % larger for those developmentally exposed to predator kairomones (ANOVA, $F_{1, 76} = 30.1624$, $P < 0.001$).

Increased temperature led to increases in shell length, width and area. Shell length of hatchlings developed at 20 °C were 10 % larger compared to those at 15 °C (Fig. 2.5. B) (ANOVA, $F_{1, 76} = 15.966$, $P < 0.001$). Width of hatchlings developed at 20 °C were 5 % wider compared to those at 15 °C (ANOVA, $F_{1, 76} = 4.3625$, $P = 0.040$). Area of hatchlings developed at 20 °C were 10 % larger compared to those at 15 °C (ANOVA, $F_{1, 76} = 5.2056$, $P = 0.025$). Temperature had no significant effect on the velum size (ANOVA, $F_{1, 66} = 0.5716$, $P > 0.05$). There were no significant interactions between temperature and the presence or absence of predation kairomone for the shell size (ANOVA, $F_{1, 76} = 0.037$, $P > 0.05$, width (ANOVA, $F_{1, 76} = 0.4239$, $P > 0.05$), velum size (ANOVA, $F_{1, 66} = 0.0254$, $P > 0.05$), or the area (ANOVA, $F_{1, 76} = 2.2529$, $P > 0.05$).

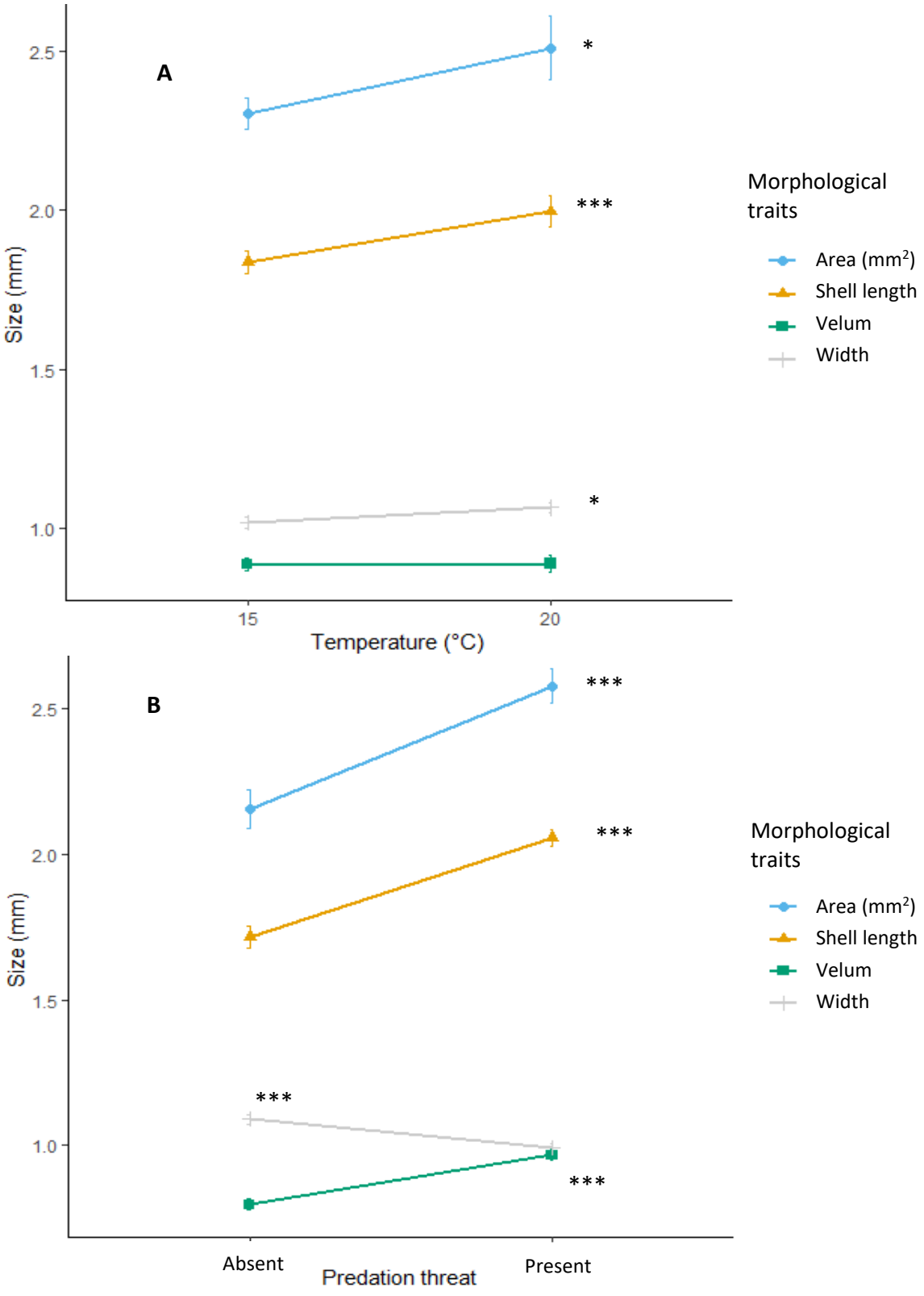

Figure 2.5 (A) The size (mm (Area mm^2)) of measured morphological traits of *Littorina littorea* developed at 15 °C or 20 °C. (B) The size (mm (Area mm^2)) of measured morphological traits of *Littorina littorea* developed in the presence or absence of predator kairomones. Data are means ±SE. * = $P < 0.05$ ** = $P < 0.01$, *** = $P < 0.001$

2.3.3 Moving or stationary swimming activity of hatchlings

There was a significant interaction between temperature and developmental predator kairomone exposure on the proportion of moving:stationary *Littorina littorea* larvae within 24 hours of hatching (linear regression, $\chi = 5.8128$, $P = 0.016$) (Table 2.1). 57 % of larvae developed in control conditions were stationary rather than moving; however, remaining treatments expressed increase in movement behaviours compared to remaining stationary. 75 % of those in predator present treatments at 15 °C expressed movement behaviours, and 77 % for those in predator absent conditions at 20 °C expressed movement behaviours; however, 85 % expressed movement behaviours for those developed in predator presence at 20 °C. There is no significant difference for the movement of larvae subject to acute exposure to predator kairomones post development, nor did it interact with temperature or developmental predator exposure.

Table 2.1 Proportion of moving larvae compared to stationary larvae when subject to predator absent conditions represented as a ratio of moving:stationary.

Development temperature (°C)	Predation Threat	
	Absence	Presence
15	6:8	15:5
20	17:5	22:4

2.3.4 Swimming speed

There was a significant interaction between acute exposure and developmental exposure to predator kairomones on the movement speeds of *Littorina littorea* larvae (linear regression, $\chi = 5.330$, $P = 0.021$) (Table 2.2). The developmental exposure to predator kairomones had a significant effect on the larval swimming speed (linear regression, $\chi = 7.025$, $P = 0.008$) (Fig. 2.6). Larvae developed exposed to predator kairomones have movement speeds approximately 1.5 times faster than those in the control treatment. Acute exposure had a smaller but still significant effect on the larval

swimming speed (linear regression, $\chi = 5.678$, $P = 0.017$), with those exposed to predator kairomones reducing movement speeds by 14 %, compared to those in predator absent conditions. There was no significant effect of developmental temperature on the larval swimming speed (linear regression, $\chi = 0.102$, $P = 0.750$). There was a positive correlation between length of the velum of hatchlings and swimming speed for larvae hatched under predator kairomones (Pearson's, r= 0.411, n = 38, P = 0.011) (Fig. 2.7), but no significant correlation for those developed under predator absent conditions (Pearson's, r = -0.068, n = 20, P = 0.768).

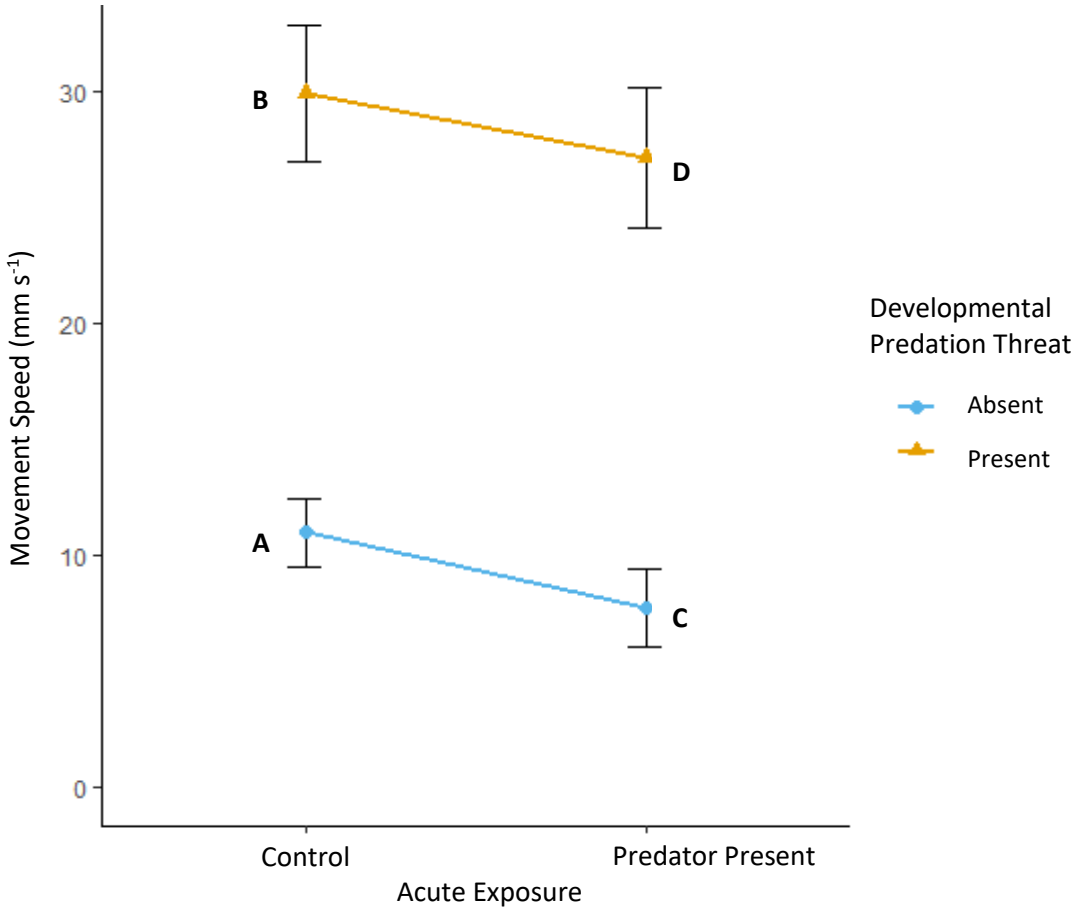

Figure 2.6 Mean movement speeds of *Littorina littorea* (mm s^{-1}) developed under predator absence or presence. Graph and data exclusive of stationary larvae. 'Predator Present' animals were subject to predation threat during video capture, whereas 'Control' animals were not. Capitalised letters are interactions of drivers for Table 2.

Table 2.2 Percentage movement speed differences for interactions using keys from Figure 2.6

Significant interactions
B is a 146% increase of A
C is a 17% decrease of A
D is a 118% increase of A
C is a 67% decrease of B
D is a 12% decrease of B
D is a 162% increase of C

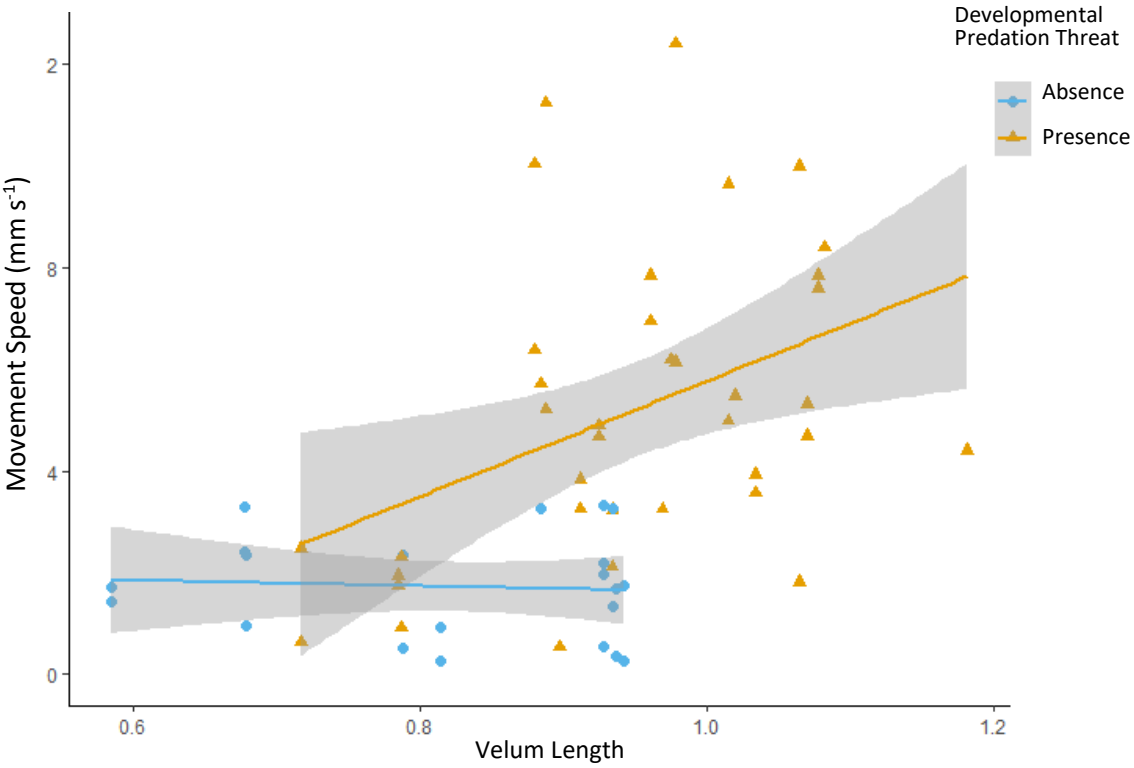

Figure 2.7 Velum length and speed of *Littorina littorea* larvae at 24 hours post hatch after developing as embryos in either the absence or presence of predatory fish kairomone. Regression lines dependant on developmental predation threat, with 95% confidence interval (shaded area)

2.4 Discussion

2.4.1 Summary of results

This study investigated the interaction between temperature and predator kairomones on the induced defence response *Littorina littorea* larvae. Temperature and predator kairomones had an interactive effect on the embryonic development time, with chronic developmental and acute exposure to predator kairomones both effecting larval movement (swimming speed) of *L. littorea.* There was no interactive effect on the morphology; however, the main effects of predator kairomones on embryo development did result in the expression of shell lengths significantly larger (20 %) than those unexposed to predation, and significantly larger velum (22 %) Velum size also has a significant positive correlation with swimming speed for those exposed to predator kairomones during development, with those exposed to predator kairomones during development also expressing faster swimming speeds. Acute predator presence showed to have a significant effect on the swimming speeds of *L. littorea* hatchlings, displaying a slight decrease in swimming speeds. The interaction of increased temperature and predator kairomones also increased the likelihood of larvae displaying active movement rather than remaining stationary. At 15 °C, embryos developed under predator presence hatched significantly faster, whereas embryos developed under predator presence at 20 °C hatched significantly slower than those developed under predator absence at 15 °C, suggesting that increased temperature due to climate change will impact the predator induced hatching response of marine larvae.

2.4.2 The combined effect of abiotic and biotic drivers on developing embryos and larvae

The study of larval stages is important when considering how an organism is affected by the environment (Thorson, 1950; Dupont, Dorey and Thorndyke, 2010). The many studies that do investigate larvae show that the rate of development is a plastic trait, that hatching rate can increase or decrease when subject to predation (Chivers *et al.*, 2001; Touchon and Wojdak, 2014), and a change in temperatures can delay or progress development (Vanhaeck and Sorgeloos, 1989; Saiah and Perrin, 1990; Weydmann *et al.*, 2015). This study has similar findings, showing that the development time is altered by increased temperature and predator kairomones, as well as the interaction of these two factors resulting in an antagonistic effect on development time. The interaction between increased temperature and predator kairomones resulted in in development time that was similar to that of embryos subject to predator kairomones alone. The increased shell and velum size of hatchlings was sustained regardless of temperature, and the antagonistic effect is likely a combination of two different mechanisms working to ensure increased size is sustained as larger body size may only able to develop at a specific rate, and this may be capping the increased development time shown by those developed under increased temperature (Coors and Meester, 2008).

The antagonistic interaction between predator kairomones and increased temperature for *L. littorea* give insight into the response of marine larvae subject to multiple stressors in projected climate conditions, as an increase in temperature has shown to have a negative effect on the induced response of developing *L. littorea* embryos. A recent study from Mira-Mendes and colleagues (2019) shows how the

interaction between increased temperature and predation threat affect the chance of survival of amphibian larvae, *Rhinella jimi*. Additional stressors on organisms have been shown to be cumulative, increasing mortality (Relyea and Mills, 2001). Larvae of *L. littorea* in the current study in have shown that when developmentally exposed to predator kairomones and increased temperature at 20 °C, they are 7x more likely to show active movement instead of stationary behaviours compared to those in absent conditions at 15 °C. This is likely due to the increased kinetic energy availability that allows for faster physiological processes (Gillooly *et al.*, 2001; Claireaux, Couturier and Groison, 2006) as well as the predator avoidance behaviours (Allan *et al.*, 2015); however, active movement was more common in larvae subject to increased temperature alone, presenting an antagonistic effect when subject to both these drivers.

2.4.3 Predation on embryo development and larval behaviour

The results of this study also show that larvae are capable of detecting predators whilst encapsulated, and not only are they capable of expressing a decreased development time, but also produce a larger velum which appears to result in an increased swimming speed; likely important for avoiding filter feeding planktivores. Acute exposure to predation threat usually results in predator avoidance behaviours such as reduced swimming speeds (Buskey, Lenz and Hartline, 2012); however, when the predator passes, the organisms can continue 'normal' behaviour, such as food consumption. When developmentally subject to chronic predation threat, the larvae express increased swimming speeds in preparation for prolonged predation pressure that would increase chances of predator avoidance and still allow for feeding behaviours (Lawrence *et al.*, 2017).

The decreased development time of *L. littorea* for those chronically exposed to predator kairomones may also be a means of predator avoidance, reducing the time in the water column as a passive particle and hatching so that the larvae can express active predator avoidance behaviours (Miner, Donovan and Andrews, 2010; Oyarzun and Strathmann, 2011). Reduced development time due to predation presence has been observed in some species of anuran larvae (Chivers *et al.*, 2001; Warkentin, 2011; Touchon and Wojdak, 2014), which can vary depending on the predator, as well as damselfish and cuttlefish embryos, which use innate chemoreception to get an understanding of predation risk outside of the egg (Atherton and McCormick, 2015; Mezrai *et al.*, 2020). When the embryos of *Nucella lamellosea* were exposed to *Hemigrapsus oregonensis*, they express an increase in development time, taking longer to hatch (Miner, Donovan and Andrews, 2010). This was also the case when *N. lamellosea* are exposed to *Idotea sp.*, where the embryos take longer to hatch; however, the combination of these two drivers had an additive effect on the development time of the embryos, further increasing the time it takes to hatch. Predator kairomones had the reverse effect on *L. littorea,* reducing the development time, likely due to the predator type. Remaining encapsulated to avoid a crushing predator and hatching faster to avoid predation by pelagic planktivore are methods of predator avoidance but comparing these two mollusc larvae would not be representative, and further investigation into predator induced hatching for *L. littorea* would give better insight into predator detection and hatching plasticity.

One of the more significant findings of this study was the behavioural response of larvae following chronic exposure to predator kairomones. Those subject to predator kairomones during development express up to 162 % faster swimming speeds

regardless of acute exposure to kairomones. This is likely due to the increased size of the velum, and therefore is a morphologically plastic trait that has led to a behavioural response. One study (Van Buskirk and McCollum, 2000) investigates the swimming performance after observing *Hyla versicolor* tadpoles with naturally occurring and surgically created tail morphologies. When *H. versicolor* are developed under predation threat (*Anax longipes),* they express plastic traits in their tail which is assumed to increase swimming performance as deeper tailed individuals have a higher survival rate when subject to predation. The study concluded that the shape of the tail did not influence the swimming performance of the tadpoles. The positive correlation between velum size and swimming speeds is only evident for *L. littorea* developed under predation pressure; larvae in predator absent conditions expressed no such correlation, even though a naturally occurring diversity in velum size existed. Chan, Jiang and Padilla (2013) suggest that changes in velum positioning through muscular control could affect larval swimming speed, without changes in the ciliary beats. This is likely to balance a possible trade-off to sustain filtering efficiency whilst increasing swimming speeds. This proposes the possibility that the change in swimming speed for *L. littorea* is behavioural, but only made possible by the increased velum size developed under predator exposure. It has been previously noted how larvae subject to increased temperature are more likely to express active movement instead of stationary behaviours. This is likely due to an increase in available energy, allowing a higher proportion of larvae to express active movement behaviours, whereas a larger proportion of those in 15 °C express similar behaviours to those in the field (Gillooly *et al.*, 2001; Ziarek *et al.*, 2011), with 15 °C being the higher end of temperatures *L. littorea* are exposed to in the field (Cooper, 1958; Hawkins *et al.*, 2017).

2.4.4 Increased temperature and the development of *Littorina littorea*

A higher rate of development under a 5 °C temperature increase was expected (Scheltema, 1967; Hoegh-Guldberg and Pearse, 1995; Oliphant, Hauton and Thatje, 2013; Pan and Herbing, 2017; Ruthsatz *et al.*, 2018), as faster development is likely explained by the Q_{10} temperature coefficient (described in section 1.3 Marine larvae and phenotypic plasticity). This coefficient relates a 10 °C temperature increase to an increase in biological processes for cold blooded organisms (Arrhenius, 1915; Davies, 1966; Robinson, Peters and Zimmermann, 1983; Heldmaier and Ruf, 1992; Gillooly *et al.*, 2001; Mundim *et al.*, 2020). Thépot and Jerry (2015) subject an Australian strain of *Lates calcarifer*, Asian Seabass, to varying temperatures during embryonic development, resulting in increased development rates correlating with increased temperature, but with 30 °C having the highest hatch rate. This study looks at a wide range of temperatures in 2 °C intervals, showing developing *L. calcarifer* subject to small temperature intervals are capable of expressing significant changes in hatch rates, and illustrating the sensitivity of marine larvae. Castelo Branco, Antas and Cunha (2014) did preliminary studies on *L. littorea* populations originating from Portugal, developing embryos in temperatures of 22 °C, a more ecologically relevant temperature for these populations, but still resulted in a faster development than anticipated based on the study done by Fish (1979), which was based on UK populations subject to highs of approximately 18 °C within the Plymouth sound, but mean temperature is more commonly approximately 16 °C during the summer months and as low as 5.3 °C in winter months (Cooper, 1958; Hawkins *et al.*, 2017). This shows that higher temperatures do lead to faster development even when populations are adapted to those climate conditions.

2.4.5 *Littorina littorea* as model pelagic larvae

L. littorea can express phenotypically plastic responses throughout all life history stages. Adult *L. littorea* are capable of expressing phenotypic plasticity when subject to predation and ocean acidification (Hadlock, 1980; Bibby *et al.*, 2007; Brookes and Rochette, 2007); and this study has shown how *L. littorea* embryos and larvae express induced defences and plasticity in response to multiple drivers. The interaction between multiple drivers is shown to be complex, with the interaction between predator presence and 5 °C temperature increase leading to a reduction in development time compared to larvae exposed to increased temperature alone. Auld, Agrawal and Relyea (2010) theorise that the expression of a phenotypically plastic trait in one environment may limit the plastic response in another. The results here show that the introduction of predator presence at 20 °C decreases the development rate of embryos under these conditions; however, the reduction in developmental rate is likely due to the increased size-at-hatch of the larvae, whereas hatchlings subject to increased temperature alone have a more similar shell length and velum size to those hatched in predator absent conditions at 15 °C.

Chevin, Lande and Mace (2010) state that the ability to remain phenotypically plastic allows organisms to adapt to unfavourable environments. In the current study, *L. littorea* have shown their ability to express phenotypic plasticity from its earliest life stages, as those subject to chronic predation threat throughout embryonic development express an increased swimming speed regardless of acute post-hatch exposure to predation threat, a response that is likely due to their larger velum size as a result of developmental chronic exposure to predation threat. Adult *L. littorea* express thicker shells when subject to a crushing predator (Rundle *et al.*, 2004; Bibby *et al.*, 2007), which

increases the effort required by the predator to crush their shells. Larval *L. littorea* developed under predator exposure develop larger velum and larger shell size, but shell thickness was not measured. It may be possible that *L. littorea* are capable of detecting predator type during development, with this response being most appropriate for planktivore predator avoidance.

Further investigation into the interactions of abiotic and biotic stressors, using *L. littorea* as a model organism, would provide a better understanding of the interactive responses in which marine larvae may possess under these conditions. An example of this may be inducing crab zoea of *Carcinus maenas*, a predator that has a different feeding method compared to the planktovoric fish used in this study. Crab zoea may induce a different plastic response, and interact differently with abiotic and biotic drivers. Not only this, but the investigation of additional abiotic drivers may provide a better understanding of how larvae may cope with predation in projected climate change.

2.4.6 Summary

This study aimed to investigate the interactions between abiotic and biotic drivers, looking at how temperature and the induced defence response of marine larvae interact when subject to a range of planktivorous fish. It should be noted, that although this study does investigate the interaction between these two drivers, investigating a more in-depth scope of continued development will highlight the impact of any carry-over effects on a more extensive coverage of *L. littorea* life history stages. Continuing the exposure to temperature drivers and predation threat throughout these life history stages, withdrawing them for each stage, will explore the interactions and carry-over effects in each stage, giving a more thorough representation of how abiotic and biotic

drivers interact on the development of *L. littorea*. Regardless of this, the study represents the morphological and behavioural plasticity expressed by *L. littorea* from very early development, with interactive responses when subject to both abiotic and biotic drivers. This study also provides a foundation for further investigation into continued development and survival of these hatchlings and their development under varying predator types.

Chapter Three: Conclusion

3.1 Introduction

This thesis set out to investigate how predation threat (fish kairomones) and thermal stress (i.e. increased temperature) affected the development and post-hatching performance of *Littorina littorea* embryos and larvae. The study conducted in Chapter 2 exposed developing *L. littorea* embryos to a factorial design of two temperatures (15 °C or 20 °C), and the presence or absence of predator kairomones (fish mucus). Chapter 2 measured the development time and morphology or embryos and investigated carry-over effects on the movement (swimming) of hatchling larvae, whilst also looking at movement under acute exposure to predator kairomones.

3.2 What does this study add to our understanding of phenotypic plasticity in marine invertebrates in response to global environmental change?

The investigation of multiple drivers is a powerful approach for understanding the phenotypically plastic responses of marine organisms, especially when considering these responses within the context of projected climate change (Przeslawski, Byrne and Mellin, 2015; Cole *et al.*, 2016; Gunderson, Armstrong and Stillman, 2016). Projected climate change would include additional stressors to consider out of the natural fluctuations experienced by some organisms (Hugget and Griffiths, 1986; Helmuth and Hofmann, 2001; Auld, Agrawal and Relyea, 2010; Gunderson, Armstrong and Stillman, 2016). However, while few studies have been conducted on the interaction of projected climate change drivers with biotic stressors, our understanding remains limited (Przeslawski, Byrne and Mellin, 2015; Gunderson, Armstrong and Stillman, 2016).

Whilst there are a select few studies that investigate the interactions between these abiotic and biotic stressors in adult marine and freshwater organisms (Berglund and Bengtsson, 1981; Claramunt and Wahl, 2000; Hansson, 2004; Munday *et al.*, 2009; Dixson, Munday and Jones, 2010; Cripps, Munday and McCormick, 2011; Nilsson *et al.*, 2012; Allan *et al.*, 2015), investigations into marine larvae are likely to give a differing result to their adult life history stages, due to the way in which drivers interact with them in the environment (Byrne and Przeslawski, 2013; Przeslawski, Byrne and Mellin, 2015). Some larvae express developmental plasticity, which has shown to influence ontogeny as well as morphology that may have carry-over effects to their adult life history stages (West-Eberhard, 2005; Simith, Diele and Abrunhosa, 2013; Przeslawski, Byrne and Mellin, 2015; Cole *et al.*, 2016; Moore and Martin, 2019). Chapter 2 shows the interactions of increased temperature and predation threat on the development of marine larvae. The study conducted in Chapter 2 adds to our understanding of developmental plasticity, showing that future projected climate temperatures have an interactive effect on the induced response of the marine larvae, with varying hatching rates expressed for developing *Littorina littorea* larvae.

The investigation of multiple drivers may allow for better insight into ecological realism (see section 1.5 Plasticity and multiple drivers) (Kraufvelin, 1999; Byrne and Przeslawski, 2013). Chapter 2 investigates multiple drivers, exposing an antagonistic response between abiotic and biotic drivers expressed by early development *L. littorea*, which would not have been exposed with the study of these drivers in isolation. This suggests Chapter 2 may provide better insight into the investigation of ecological realism. There still remains the fact that insight into true ecological realism is a contentious idea, due to the many factors and fluctuations in which organisms may face

in situ (Kraufvelin, 1999; Letellier and Aziz-Alaoui, 2002; Przeslawski, Byrne and Mellin, 2015); however, the selection of key relevant drivers aids a study to investigate an artificial system that holds some similarity of those *in situ*. Temperature, being one of the key drivers of climate change (Laffoley and Baxter, 2016; Iverson *et al.*, 2020) was selected for the study in Chapter 2. Sea surface temperature are projected to rise by between 1 – 4 °C by 2100 (Laffoley and Baxter, 2016), and embryos of the intertidal mollusc, *L. littorea,* remain in shallow pelagic waters during development where they will be exposed to projected temperature increase. Here, in the pelagic zone, they are likely to be predated upon by a range of planktivorous fish. The selection of drivers used in Chapter 2 was based on these drivers, as *L. Littorina* are highly likely to be subject to them, and Chapter 2 shows that the selected drivers have a separate and interactive effect on various developmental measures, representing their importance for the development of *L. littorea.*

3.3 Does the marine larval response to predation exposure fall in line with theory?

Predation stress can induce phenotypic traits that aid predator avoidance (Tollrian and Harvell, 1999). This is suggested to be a form of active plasticity (see section 1.4 Induced defences in marine larvae) (Whitman and Agrawal, 2009) due to the complex phenotypic response of the organism expressing them. We can see in Chapter 2 that size at hatch for *Littorina littorea* larvae is larger for those exposed to predator kairomones during development, regardless of temperature or hatching time. Size at hatch may be indicative of faster development which may reduce time to settlement stages, increasing predator avoidance (Spight, 1976; Oyarzun and Strathmann, 2011). With a larger size at hatch, the study in Chapter 2 shows that the velum size is larger

which correlates positively with the swimming speed of individuals and further aids predator avoidance (Spight, 1976; Oyarzun and Strathmann, 2011; Chan, Jiang and Padilla, 2013). This shows that the increased size of *L. littorea* is an active induced plastic response, as the increase in size also occurred when subject to an increase in temperature, and there was a delay in development time to allow for this increase in size.

With adult *L. littorea* expressing shell thickening when subject to predator exposure (Kemp and Bertness, 1984; Ruth *et al.*, 2007), it was theorised that their larval life history stages may have been capable of expressing early developmental plasticity when subject to predator exposure. This theory was confirmed when conducting the study in Chapter 2. The induced defences expressed by the larvae were reduced development time, increased size at hatch and faster swimming speeds, all defences that may aid with planktivorous predator avoidance (Cowan, Houde and Rose, 1996; Teplitsky *et al.*, 2005; Vaughn, 2010; Van Donk, Ianora and Vos, 2011; Warkentin, 2011). Functional predation-specific induced defences have been compared in a freshwater organisms (Teplitsky *et al.*, 2005; Hoverman and Relyea, 2009; Hettyey *et al.*, 2011; Bourdeau and Johansson, 2012), where the defences match the predation type. In *Littorina scutulata*, larvae express smaller operculum and more rounded shells when exposed to *Cancer spp.* zoea during development (Vaughn, 2007), an induced defence that limits zoea spine protrusion onto soft body tissues. With *L. scutulata* and *L. littorea* expressing differing induced responses to different predators, there may be a possibility for functional predator-type induced defences, but this would require further investigation using the same species and varying predators to determine whether marine larvae can express functional predator type responses.

With the study conducted in Chapter 2 showing the larvae prioritising the expression of larger size-at-hatch over decreased development time, there is likely a trade-off with the increased size aiding predator avoidance more than shortened development (Oliphant, Hauton and Thatje, 2013; Touchon and Wojdak, 2014; Mira-Mendes *et al.*, 2019). However, projected climate change incorporates many abiotic and biotic factors that are likely to interact (Przeslawski, Byrne and Mellin, 2015; Gunderson, Armstrong and Stillman, 2016). Investigating additional drivers will provide a better understanding of the interactions of drivers on marine larvae in projected climate conditions.

3.4 Temperature as a key driver in a multiple driver study

Temperature is a significant driver related to climate change, with sea surface temperature expected to rise 1 – 4 °C by 2100 (Barrows *et al.*, 2007; Thompson, 2010; Laffoley and Baxter, 2016). Many studies have represented an understanding of how temperature may affect biological processes of marine organisms, and have deemed it tone if the key determinants of the phenotype (Iverson *et al.*, 2020). With temperature, it was assuming that there would be an increase in most, if not all, processes studies in Chapter 2, in correlation with the Q_{10} temperature coefficient (Arrhenius, 1915; Knies and Kingsolver, 2010; Mundim *et al.*, 2020).

Development rate of *Littorina littorea* did follow the Q_{10} temperature coefficient, with embryos chronically exposed to increased temperature expressing an increased development rate (Chapter 2) and hatching sooner than those in control conditions. Increased temperature didn't lead to a larger velum size; however, other morphological traits measured were larger in comparison to those exposed to control conditions, which has been shown to occur in a few studies (Ghosh, Testa and Shingleton, 2013;

Przeslawski, Byrne and Mellin, 2015; Weydmann *et al.*, 2015), as well as smaller morphologies in others (Laurel *et al.*, 2008; Quinn *et al.*, 2013; Przeslawski, Byrne and Mellin, 2015). The expression of larger morphologies for *L. littorea* in higher temperatures may have a physiological advantage (Ghosh, Testa and Shingleton, 2013; Weydmann *et al.*, 2015), but further investigation into the carry-over effects of the temperature-size relationship expressed by *L. littorea* will give insight into what advantages, or disadvantages, may be.

3.5 Using *Littorina littorea* as a model

With marine larvae being one of the more significant developmental methods of marine organisms (Thorson, 1950; Dupont, Dorey and Thorndyke, 2010), studies that investigate factors that interact with development may indicate several carry-over factors to their adult life-history stages (Hoegh-Guldberg and Pearse, 1995; Simith, Diele and Abrunhosa, 2013; Przeslawski, Byrne and Mellin, 2015; Cole *et al.*, 2016; Parker *et al.*, 2017; Moore and Martin, 2019). Marine larvae are the basis of many ecological communities (Gaines and Roughgarden, 1985; Hoegh-Guldberg and Pearse, 1995; Van Buskirk, 2002; Byrne and Przeslawski, 2013), and so investigating how projected climate change interacts with their development as well as their ability to produce an induced defence response is key to understanding the structure and survival of future communities (Przeslawski, Byrne and Mellin, 2015).

The study conducted in Chapter 2 uses larvae of *L. littorea* as model organisms for marine larval studies. One of the main advantages of using *L. littorea* was the ease of laboratory handling (Newell, 1958), with regular spawning during the breeding season (Fish, 1972; Grahame, 1975), well studied embryo collection (McCoy *et al.*, 2020) as well as accessible development monitoring techniques. This allowed for embryos to be

collected very early in development, which aided in the study of chronic developmental exposure to stressors, where embryos could be individually monitored.

The spawning of *L. littorea* embryos in Plymouth, UK, lasts from around January to June (Fish, 1972), which allowed for the run time of pilot studies, as well as the main study, but did result in minimal time for any complications that may arise. Complications experienced in the study of Chapter 2 included embryo parasites originating from the adult population, as well as adult lab acclimation that resulted in premature reduced/inhibited spawning due to long acclimation to lab environments. Both complications resulted in the need for new spawning populations, and when the study was conducted at the end of May, it was difficult to locate remaining spawning adults with only one remaining sample location in Cawsands Beach, UK (50.331058, -4.201766).

Another advantage of using *L. littorea* as a model organism is their development, which allows for investigation into carry-over effects in a relatively short timescale. With pelagic embryos developing into pelagic swimming larvae, it allows for a study to investigate over two distinct life history stages within a 2-week timescale. The study conducted in Chapter 2 shows that chronic exposure to predator kairomones leads to carry-over effects into the post-hatch larval stages of *L. littorea*.

Chapter 2 confirms that there is an interactive response to increased temperature and predator exposure on the development of marine larvae, which shows to pose some carry-over effects to their future development (Hoegh-Guldberg and Pearse, 1995; Simith, Diele and Abrunhosa, 2013; Ituarte *et al.*, 2014), which may affect adult performance and, therefore, the community structure of *L. littorea* (Moore and Martin, 2019), but further investigation is required to see how larvae develop under

further chronic exposure until settlement and adulthood, when predators change and the organisms have more control over their time within the water.

With many studies investigating the interactions of multiple stressors in the marine environment (Coors and Meester, 2008; Przeslawski, Byrne and Mellin, 2015; Gunderson, Armstrong and Stillman, 2016), and a select few investigating the interaction this has on larval development (Byrne and Przeslawski, 2013; Przeslawski, Byrne and Mellin, 2015), the study (Chapter 2) adds to our knowledge by looking at the interactions of abiotic and biotic drivers, focussing on the interactive effect this has on embryonic development as well as giving insight into carry-over effects in hatchling larvae due to relatively short development times. It confirms the impact that projected climate change will have on larval organisms and proposes an impact for ecological communities and the future of marine ecosystems. Further investigation would be required to gain more understanding of carry-over effects of these drivers in further developed organisms.

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Appendecies

Appendix 1

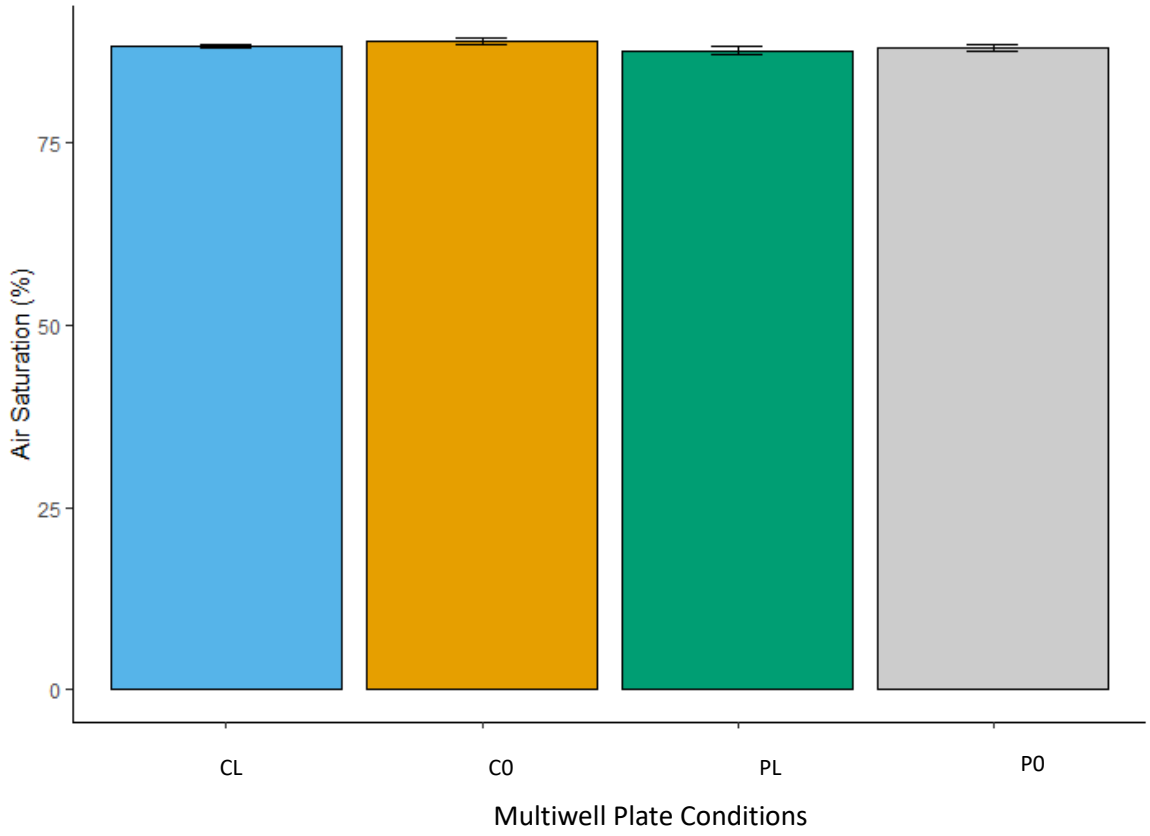

Figure 3.1 Mean air saturation (%) throughout pilot study, with mean values on each group. ±SE (CL) Control with larva, (C0) Control without larva, (PL) Predator presence with larva, (P0) Predator presence without larva. ±SE

Pilot studies were conducted to measure whether oxygen levels were affected by the addition of predator cues and investigating air saturation levels within the 96

multiwell plates. Four PreSens Oxygen Dots (PreSens Gm bH, Regensburg, Germany) were placed in each 96 multiwell, with two dots in predator exposed conditions and two in predator absent. For each of these conditions, there was one well containing an embryo and one 'dummy' well with no embryo. Plates were sealed using Aeraseal (Excel Scientific, Inc.) and covered with an inverted 3 litre plastic aquarium. They were maintained at either 15 °C or 20 °C in separate constant temperature (CT) rooms. Using a Fibrox Presens (PreSens GmbH, Regensburg, Germany), the oxygen levels of wells containing a PreSens Oxygen dot was measured every 24 hours for two weeks. Air saturation levels were analysed in RStudio (RStudio Team, 2016) using the outlier function, which removed anomalous data due to human error and PreSens calibration. The data shows that air saturation is sustained above 85 % (Fig. 3.1), with freshly changed water starting around 90 %. Therefore, the organisms in each developmental treatment would remain normoxic during the study if these protocols are sustained.

Appendix 2

Velum length was used for a proxy of velum area. Velum area was collected from a few images, as there were fewer images where the velum area could be obtained by tracking the outline of the velum and calculating the area using ImageJ. A subset of samples were statistically analysed in RStudio (RStudio Team, 2016), using Pearson's product-moment correlation coefficient between velum length an velum area. The test showed that there is a positive correlation between velum length and velum area ($r=0.871$, $n=21$, $P < 0.001$). This positive correlation verifies that velum length can be used as a proxy for velum area.

Appendix 3

R Studio code (R Core Team, 2017) for Gamma GLM analysis

```
tracksize <- read.csv("C:/Users/sebutt/OneDrive - University of Plymouth/Documents/ResM/1 - Personal Research/data/1. Data in use/tracksize.csv")

tracksize$treat<-as.factor(tracksize$treat)

tracksize$temp<-as.factor(tracksize$temp)

tracksize$dev<-as.factor(tracksize$dev)

require("lme4")

anovats <- glm(mms ~ area + treat*temp*dev, data = tracksize, family = Gamma())

summary (anovats)

#plot(anovats)

step(anovats)

glmstep<-glm(formula = mms ~ width + treat + temp + dev + treat:dev,

        family = Gamma(), data = tracksize)

Anova(glmstep, type=c("3"))
```