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Promoting phenotypic plasticity to improve aquaculture and conservation practices of the European lobster (*Homarus gammarus*; Linnaeus, 1758)

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Structure of the thesis

The aim of this thesis was to understand how early-life exposure to different hatchery-rearing conditions alters the development of plasticity in ecologically relevant phenotypic traits, and to assess the potential implementation of this plasticity for enhancing the success of conservation programs. For this purpose, I investigated the expression of phenotypic plasticity in juvenile lobsters (*Homarus gammarus*) subjected to different rearing conditions. In the introduction (Chapter 1), I described the general aims of the research and the study system. In Chapter 2, I presented literature research on marine organisms commonly involved in conservation aquaculture programs, providing a comprehensive exploration of phenotypic plasticity and its implications for conservation biology. In Chapter 3 I investigated whether the size of the individual rearing compartments affected the phenotypic responses of juvenile lobsters during their early benthic stages. In Chapter 4, I compared plasticity expression between juveniles exposed to different degree of environmental complexity and exposure to predatory cues. In Chapter 5, I discussed the implications of the results, their relevance in conservation biology and future perspectives. The above research activity led to 2 research papers, one of which has already been published in a peer-review scientific journal while the other is currently published as a preprint.

Abstract

Organisms can adapt to the modified pressures of captive conditions either through genetic changes over generations or through phenotypic plasticity occurring within an individual's lifetime. These adaptive mechanisms can lead organisms held in captivity to develop phenotypic traits that differ from those observed in their wild counterpart. In the context of conservation aquaculture, where broodstock consist of wild animals and juveniles held in controlled conditions for a short period of time, it's highly likely that captivity induced phenotypic traits reflect an organism's ability to respond phenotypically to the rearing environment rather than genetic differences between hatchery and wild organisms. This plasticity suggests potential for change with adjustments to rearing protocols, enabling researchers to mould phenotype with consistent and reinforced cues, thus eliminating maladaptive traits and better preparing animals for life in the wild. Here, we test such an approach by investigating whether and how early-life exposure to different environmental conditions alters the development of plasticity in ecologically relevant traits (behaviour, morphology, and life-history) in European lobsters (*Homarus gammarus*)—one of the most harvested species in the Mediterranean Sea, which has been subjected to conservation programs for decades. By accessing one of the largest lobsters' hatcheries in Italy, we used the progeny of wild-caught females and manipulated—in a full factorial design—the size of their individual enclosures, the environmental complexity of these enclosures (presence/absence of substrate and/or shelter), and the level of exposure to predatory cues. We repeatedly quantified activity, risk taking, and aggressiveness of the individuals throughout their early development, capturing both mean and individual-level variation across treatments. We also repeatedly assessed life-history traits that are under strong selection in this species (carapace length, intermoult period, and growth rate). Our results offer solid evidence that effects of standard hatchery settings extend far beyond mean changes in the behaviour and life history of the animals, compromising the development of individuality and plasticity in those traits that are essentials for populations to survive in the wild, and are likely to reduce the effectiveness of conservation programs. These findings contribute to our understanding of phenotypic plasticity in marine organisms and its relevance in conservation and reintroduction programs, especially within the context of aquaculture and stock enhancement efforts for decapod crustaceans. Future research on the effects and potential disparities between captive-reared and wild animals, coupled with investigations into the effects of both inter- and intra-individual phenotypic variation among

reared organisms, will provide further insights for a correct management and planning of conservation activities.

1. Introduction

Phenotypic plasticity allows individuals to rapidly express different phenotypic traits during their lifetime in response to varying environmental conditions (Pigliucci, 2005; Reusch, 2014; Sommer, 2020), while local adaptation involves the evolutionary development of genetic variations over generations, enabling individuals and populations to thrive in a specific local environment (Kawecki & Ebert, 2004; Fan et al., 2016). Both mechanisms are adaptive strategies that contribute to an organism's success in coping with environmental challenges, with phenotypic plasticity occurring on a shorter timescale within an individual's life, and local adaptation unfolding over longer periods through genetic changes in populations (Chevin & Hoffman, 2017). Similarly, animals growing up in captivity, where selective pressures are drastically altered, may develop, through phenotypic plasticity or genetic changes, traits that differ from those observed in the wild (Araki et al., 2008; Chittenden et al., 2010; Johnsson et al., 2014; Gering et al., 2019). These cultured phenotypes may be beneficial under controlled conditions but may reduce the individual fitness when exposed to another environment (e.g. the wild environment following release). Traditionally, effects induced by captivity have been investigated almost exclusively in terms of genetic changes through natural and artificial selection (Clutton-Brock, 1992; Woodworth et al., 2002; Frankham, 2007; Robert, 2009). However, recent interest in conservation and reintroduction programs, where juveniles are held in rearing settings for a limited period before being released into the natural environment (Welcomme & Bartley, 1998; Lorenzen et al., 2010), has led to a greater appreciation of the role of developmental plastic responses (Price, 2002; Mason et al. 2013; Gering et al. 2019). In this context it’s highly likely that phenotypic alterations, occurring within a single captive generation, reflect an organism’s ability to respond phenotypically to the rearing environment rather than genetic differences between cultured and wild organisms (Gering et al., 2019; Daly et al., 2021). Nevertheless, little is known about the mechanisms of plasticity that cause phenotypic change in controlled conditions. A possible strategy to assess whether the developmental environment can generate an environment-specific phenotype is to manipulate the developmental conditions, either by rearing animals in a fluctuating setting, or by exposing them to challenging conditions that mimic the difficulties experienced in their natural habitat.

In the context of aquaculture, conservation programs typically involve the production in captivity of aquatic species for the restoration of depleted populations, the reintroduction of endangered species, or the enhancement of wild stocks (Froehlich et al., 2017; Bell et al., 2008).

Specifically, stock enhancements require the capture of ovigerous females in the wild and their maintenance in controlled, safe, settings until egg hatching, so that offspring are reared and released in the wild (Hamasaki & Kitada, 2008). For this kind of conservation programs, marine decapod species are considered excellent candidates: they are characterized by high fecundity but relatively low rates of recruitment (i.e. larval mortality in the wild is typically high), they are subject to fishery overexploitation due to their high commercial value, they are easily accessible to harvest and they play a fundamental role in marine trophic webs, being omnivorous and prey for many predators (Daly et al., 2021; Boenish et al., 2022). However, changes in the natural, sexual and artificial selective pressures that enhance fitness in rearing environment can lead to a directional shift in phenotypic traits away from the wild phenotype towards an optimal mean trait value for captive conditions, compromising the success of conservation actions (McDougall et al., 2006; McPhee & McPhee, 2012). Specifically, in-hatchery selective pressures can induce the development of traits that are maladaptive in the natural environments, for example impairing feeding, interspecific (e.g. predator avoidance, parasite infections, competition) and intraspecific interactions (e.g. dominance, courtship, and mating), in addition to physiological, behavioural and morphological abnormalities (e.g. low immune functions, lack of sexual and defence/offense skills, poor cognitive abilities, modified body and limb form; Braithwaite & Salvanes, 2005; Salvanes et al., 2013; Naslund, 2021). Additionally, standard hatchery procedures, often characterized by uniform and unchallenging environments, may also lead to reduced genetic diversity and phenotypic variation, altering at the same time the capacity for plasticity of reared organisms (Mathews et al., 2005; Crispo, 2008; Frankenhuis & Panchanathan, 2011). Thus, from a conservation perspective, producing organisms very similar to each other and not capable of facing rapidly changing conditions after their release in the wild may result in poor conservation programs (Snyder et al., 1996; Fischer & Lindenmayer, 2000; O'Regan & Kitchener, 2005). One potential approach to prevent and avoid the development of anomalous and undesirable traits and to enhance the expression of phenotypic variation, is to promote phenotypic plasticity by exposing reared organisms to ecologically-relevant cues (Johnsson et al., 2014; Daly et al., 2021).

This research aims to understand the mechanisms capable of eliciting phenotypic plasticity in a controlled environmental setting, in order to promote the development of wild-like phenotypes and to increase their overall variation, likely to improve the effectiveness of conservation programs. Specifically, we focused on the European lobster (*Homarus gammarus*), one the most harvested species in the Mediterranean Sea, which has been subjected to stock-enhancement programs for

decades. By studying the phenotypic effects arisen from the exposition of juvenile lobsters to different rearing conditions, we have made inferences on how phenotypic plasticity can be used to enhance the expression of favourable traits essential for survival in the natural environment.

To better understand the implications of phenotypic plasticity in conservation aquaculture programs, the research activity was structured as follows. First, I conducted a literature review on marine species of interest for conservation aquaculture with the aim to understand how phenotypes can change in response to captive rearing conditions and with the intent of developing a conceptual framework that underscores the role of plasticity in shaping phenotypic traits in response to different environmental stimuli. In the second part of the review, I analyzed how training intervention and management strategies can mitigate the phenotypic costs of captivity and boost reintroduction success, suggesting key research questions whose answers could improve the success of future reintroduction efforts. To define the experimental design, I relied on previous studies to identify which are the main environmental factors capable of eliciting plastic responses in functional traits (behaviour, morphology, and life-history) in hatchery-reared juvenile lobsters. Then, I conducted laboratory experiments to explore how manipulating environmental conditions in captivity, in terms of size of individual enclosures, degree of environmental complexity and exposure to predatory cues, can be used to promote phenotypic plasticity and the potential for inducing the expression of favourable phenotypic traits. The experimental phase was conducted over the first 24 weeks of juvenile's benthic stage, from February to July 2021. During this period, juvenile lobsters were repeatedly subjected, at each growth moult, to behavioural tests and morphometric measurements with the aim of assessing any alterations in phenotypic development attributable to different rearing conditions. At the end of the experimental phase, I focused on data extraction, and I performed statistical analyses using a multivariate approach and analyses of variance to determine the effects of the environment (phenotypic plasticity). In the final phase, I wrote two papers, one published in a peer-reviewed scientific journal and the other published as a preprint; these papers were then included as central chapters of this thesis.

2. Exploring phenotypic plasticity in conservation aquaculture: insights, challenges, and future directions

Abstract

Phenotypic plasticity, which allows single genotypes to rapidly express diverse phenotypes in response to changing conditions, enables individuals and populations to persist in highly dynamic and fragmented environments. In addition, because of its unique ability to generate an immediate phenotypic response to the environment, phenotypic plasticity plays a key role in fostering divergent phenotypes within populations and subsequently driving diversification. These issues are particularly relevant in the context of conservation and reintroduction programs, where rearing environments, inducing distinct selection pressures, lead to directional shifts in phenotypic traits. Identifying the evolving phenotypic traits and elucidating the specific mechanisms associated with these changes in captivity can assist managers in refining strategies employed in rearing settings and reintroduction programs, simultaneously improving the prospects of post-release survival. By relying on the available literature focusing on marine organisms commonly involved in conservation aquaculture programs, we provide a comprehensive exploration of phenotypic plasticity and its implications for conservation biology. The developed conceptual framework underscores the role of phenotypic plasticity in (i) inducing phenotypic changes and reducing phenotypic variation under rearing conditions, (ii) mitigating these effects through the introduction ecologically relevant stimuli in the rearing environments, (iii) providing enhancing strategies employed in rearing settings to improve the success of reintroduction efforts. Finally, a number of relevant questions for future research are proposed.

Phenotypic plasticity in hatchery-reared organisms

Whether organisms are able to deal with environmental changes and fluctuating conditions depends on either local adaptation or phenotypic plastic responses. While local adaptation involves genetic differentiation across generations producing long-lasting phenotypes specific to each environment (Kaweki & Ebert, 2004; Fan et al., 2016), phenotypic plasticity offers a more rapid response, allowing single genotypes to readily express different phenotypes under diverse environmental conditions (West-Eberhard, 2003; De Witt & Scheiner, 2004; Pigliucci, 2005; Chevin & Hoffmann, 2017). Through these mechanisms, animals are able to adapt even to controlled conditions, such as those found in rearing environments, where modified selective pressures lead to the development of phenotypes that differ from those observed in the wild (Fleming et al., 1994; Araki et al. 2008; Chittenden et al. 2010). These cultured phenotypes can be the result of genetic changes brought about through both intentional and unintentional selection (Einum & Fleming 2001; Hutchings & Fraser 2008; Solberg et al., 2013), or the product of a plastic response to different rearing conditions (Imre et al., 2002; Skjæraasen et al., 2008; Daly et al., 2021). The degree of phenotypic change, and its permanence, are both a function of the time an individual has spent in controlled conditions (Pakkasmaa et al., 1998; von Cramon-Taubadel et al., 2005), as well as the degree of genetic change from the ancestral lineage due to captivity (Fleming et al., 1994; Blanchet et al., 2008; Hutchings & Fraser 2008; Fraser et al., 2019). Thus, minimizing generations in captivity is an effective way of minimizing genetic adaptation to rearing conditions. For example, increased growth rate and reduced behavioural complexity may result from phenotypic plasticity and become apparent within weeks or months of an organism's transfer from a natural to a rearing environment but become more pronounced in successive generations due to inadvertent selection and genetic assimilation (Lorenzen, 2000; Metcalfe et al., 2003; Huntingford, 2004; Hutchings & Fraser 2008). In many conservation aquaculture programs, where broodstock consist of wild animals and juveniles held in captivity for a very short period of time (Welcomme & Bartley 1998; Lorenzen et al., 2010), it's highly likely that phenotypic alterations reflect an organism's ability to respond phenotypically to the rearing environment rather than genetic differences between hatchery and wild organisms (Gering et al., 2019; Daly ey al., 2020). Therefore, from a conservation perspective it is fundamental to understand how phenotypic plasticity allows reared organisms to adapt to captive environments and in turn, whether and how this plasticity can be employed to better prepare them for release into the natural environment.

By providing insurance against extinction (Dobson & Lyles, 2000) and helping bolster or reestablishing wild populations (Ewen et al., 2012), the breeding of threatened species for subsequent release is a central tool in conservation biology. In the context of aquaculture, conservation programs typically involve the production in hatchery-rearing settings of aquatic species for the restoration of depleted populations, the reintroduction of endangered species, or the enhancement of wild stocks (Bell et al., 2008; Froehlich et al., 2017). However, standard hatchery procedures are often improper to guarantee a wild-like development of the individuals because they are meant to optimize growth and reproduction, rather than mimic challenging conditions from the wild (Näslund, 2021). Thus, changes in the natural, sexual and artificial selective pressures that enhance fitness in captivity can lead to a directional shift in phenotypic traits away from the wild phenotype towards an optimal mean trait value for hatchery conditions (McDougall et al., 2006; McPhee & McPhee, 2012). Such phenotypic alterations induced by rearing conditions can have multiple ramifications such as impairing feeding, interspecific (e.g. predator avoidance, parasite infections, competition) and intraspecific interactions (e.g. dominance, courtship, and mating), as well as inducing physiological, behavioural and morphological abnormalities (e.g. low immune functions, lack of sexual and defence/offense skills, poor cognitive abilities, failed claw differentiation; Braithwite & Salvanes, 2005; Salvanes et al., 2013; Näslund, 2021). In addition, uniform and unchallenging rearing environments, may also contribute to a decrease in genetic diversity and phenotypic variation, simultaneously impacting the capacity for plasticity of reared organisms (Briscoe et al., 1992; Mathews et al., 2005; Crispo, 2008; Frankenhuis & Panchanathan, 2011). These challenges arising from the modified selective pressures within hatchery environments may result in poor reintroduction success (Snyder et al., 1996; Fischer & Lindenmayer, 2000; O'Regan & Kitchener, 2005). Thus, to improve the effectiveness of such programs, animals need to have or quickly obtain, the life skills or ecological competence, necessary to survive, grow and reproduce in the wild (Suboski & Templeton, 1989; Daly et al., 2020). These objectives can be achieved by introducing ecologically-relevant environmental cues in the rearing environment, which can promote phenotypic plasticity and thus increase the potential for expressing favourable traits crucial for thriving in the natural environment (Monaghan, 2008; Daly et al., 2020; Crates et al., 2022).

Given the current anthropogenic pressure on aquatic ecosystems (Young et al., 2016), conservation aquaculture programs will likely continue to sustain and reestablish wild populations (Lema et al., 2021). Thus, efforts to understand whether and how manipulating hatchery conditions can be used to promote phenotypic plasticity and the potential for inducing the expression of

favourable phenotypic traits of reared species, have become more urgent. Experimental conditions, where environmental factors can be easily controlled and manipulated by operators and where multiple confounding factors (e.g. climate, genotype, life history stage, biotic and abiotic feedbacks from short to long timescales) can be teased apart, provide an ideal setting to identify the phenotypic traits that change and the specific mechanisms (i.e. the biotic and biotic factors) that induce plastic responses in those traits (Norberg et al., 2001; Khaw et al., 2009). Shedding light on these aspects is critical for managing risk, ensuring the effectiveness of rearing programs, and is also an important animal welfare consideration (Webster, 1995; McDougall et al., 2006). This in turn can have implications for the way rearing environments are designed and managed to ensure animals are treated ethically in captivity and post-release. By relying on experimental studies conducted on marine organisms which have been the subject of several conservation aquaculture programs, we provide a comprehensive overview of the concept of phenotypic plasticity and its relevance to conservation biology. Specifically, we first explore how hatchery environment can alter animal phenotypes, particularly in terms of behavioural and morphological traits. We then discuss how conditioning strategies, such as sensory stimulation, prerelease training and exposure to natural features, can mitigate the phenotypic costs of captivity and boost reintroduction success. Finally, we pinpoint key research questions whose answers could improve the success of future reintroduction efforts.

Captivity-induced phenotypic changes

Hatcheries rarely attempt to mimic natural conditions, but rather seek to maximize production by limiting the mortality and optimizing the growth, while minimizing the cost. In doing so, they alter the natural, sexual and artificial selective pressures experienced by reared animals, leading to a directional shift in phenotypic traits away from the wild phenotype towards an optimal mean trait value for captive conditions (McDougall et al., 2006; McPhee & McPhee, 2012). This directional shift can occur relatively quickly and reflect an organism's ability to respond phenotypically to the rearing environment, rather than genetic differences between hatchery and wild organisms (Gering et al., 2019; Daly et al., 2020). Understanding how rearing conditions influence the expression of phenotypic traits and their variation within the captive population is crucial for determining if, when, and how rearing programs should be employed for conservation purposes. To shed light on the

potential negative consequences of standard hatchery protocols, we'll highlight some maladaptive outcomes and underscore their impact on the success of conservation strategies.

Behavioural (mal)adaptations

Released individuals require a wide range of behavioural skills and cognitive abilities that depend, in part, on the environments in which they are reared and their immediate pre-release experience (Shepherdson, 1994; Rabin, 2003). Nonetheless, if the conditions provided in the rearing environment are unfavourable or lack ecologically relevant stimuli, captive animals can develop abnormal behaviours that are not observed in their wild counterpart, and which may impair their ability to survive in the wild (Huntingford, 2004; Le Vay et al., 2007). In addition, behavioural syndromes, defined as a suite of correlated behaviours expressed either within a given behavioural context or across different contexts (Sih et al., 2004), can lead to the unintentional alteration of a wide range of behavioural traits of hatchery reared animals (Huntingford & Adams, 2005; Lee & Bereijikian, 2008). Overall, erosion and divergence from wild behaviours can occur very quickly (Courtney Jones et al., 2017) and can have a predominant impact on individual fitness affecting the viability of captive source populations, and/or the probability of reintroduction success (Fischer & Lindenmayer, 2000). In this setting, our focus is on predator avoidance, cognition, and space utilization, recognized as crucial behaviours that play a significant role in the success of reintroduction programs and for which a more comprehensive knowledge base is available (Young et al. 2008; Reading et al., 2013).

Predator avoidance

One of the main behavioural issues for stocked animals is suboptimal antipredation behaviours, often acting from the first instants post-release. Antipredation behaviour is crucial for survival in the natural environment, but also potentially costly, as time spent not feeding (or reproducing) could decrease overall fitness (Brown, 2003). Although certain responses are innate (Kelley & Magurran, 2003), extensive research conducted over decades has revealed that antipredator behaviours are to some extent acquired, and hatchery-reared individuals frequently exhibit distinct reactions to potentially dangerous situations compared to their wild counterparts (e.g., Stoner, 1994; Olla et al., 1998; Einum & Fleming, 2001). For example, newly-released hatchery reared white seabream (*Diplodus sargus*) tend to ignore areas of refuge, suggesting a lower chance of avoiding predation

(D' anna et al., 2004) and wild caught seabass juveniles (*Dicentrarchus labrax*) remain immobile for longer after a simulated predatory attack than do hatchery-reared fish (Malavasi et al., 2008). Thus, the absence of predator stimuli in rearing environments is likely to result in an insufficient range of behaviours for dealing with predators (e.g., Malavasi et al., 2004; Karplus et al., 2006; Gristina et al., 2011), leading to significant losses before the released animals acquire the ability to react appropriately. Although it is quite challenging to define predation mortality rates during the post-release period, some studies have successfully achieved this goal. Sudo et al. (2008) found a mortality of 68%–83% over the first week after releasing 50,000 juvenile hatchery-reared olive flounders (*Paralichthys olivaceus*), which was mainly caused by crabs and predatory fish in the release area. It has also been demonstrated that almost half of the Atlantic salmon (*Salmo salar*) stocked into streams, due to an impaired antipredator behaviour, may be consumed by piscivorous fish within the first 2 days of stocking (Henderson & Letcher 2013).

Cognition and space use

In captivity, animals may not have the same opportunities for problem-solving, foraging, space use and other cognitive challenges they would experience in the wild. This can lead to a decline in cognitive skills, potentially affecting their ability to adapt to new or changing environments (Reading et al., 2013). If reared animals fail to exploit the habitat effectively due to poor responses to environmental cues, survival and growth are likely to be compromised (Huntingford et al., 2012). Several studies highlight the existence of cognitive differences among animals reared under different hatchery conditions (Brockmark et al., 2010; Strand et al., 2010; Salvanes et al., 2013; Cogliati et al., 2019), indicating that the rearing environment plays a part in cognition, at least in the short-term. For example, Arechavala-Lopez et al. (2020) showed that poor enriched rearing conditions impairs exploratory behaviour, spatial orientation and learning capability of gilthead seabream (*Sparus aurata*). Furthermore, captive Atlantic salmons (*Salmo salar*) that were reared in a barren environment showed less neural plasticity and inferior spatial learning ability than conspecifics reared in a more complex environment (Salvanes et al., 2013). Another relevant factor in the hatchery settings that may affect spatial cognition and exploratory behaviour is the available space furnished. For instance, juveniles' European lobster (*Homarus gammarus*) reared individually in restricted space move less and spend less time hiding in the refuge than conspecifics reared in wider spaces (Latini et al., 2023). These studies highlight that domestication and captive rearing can have profound effects on many aspects of the biology of marine animals, so it is important to

establish the extent to which cognition and space use are affected. However, cognitive traits are typically only possible to investigate in laboratory environments and there is yet little knowledge about how individual cognitive traits affect performance in nature for animals in general (Cauchoix et., 2020). More studies are needed to demonstrate whether and how cognition and space use abilities differ between cultured and wild individuals, and eventually, how these differences may affect their performance in the wild.

Morphological and growth alterations

The speed at which morphological changes occur largely depend on when the phenotype is induced and how long the environmental conditions remain altered (Miner et al., 2005). There is abundant evidence demonstrating that organisms in captivity often exhibit significant changes in their body form and growth parameters when compared to their wild counterparts (Svåsand et al., 1998; Parkes et al., 2011; Diaz-Gil et al., 2020; Herron et al., 2023). These differences may arise from shifts in intensity and targets of captive selection, favouring morphological traits that optimize fitness in such settings (Crates et al., 2022). The examples discussed below focus on how morphological traits and growth performances can change in response to hatchery conditions and how these alterations can impact an individual's chances of survival upon release, either by having effects on an individual's ability to defend against predators (avoidance and defence), ability to capture food, and/or ability to compete for resources.

Changes in morphological traits

Sometimes factors related to the hatchery environment, such as absence of predators, uniformity in substrate colour, and different food, may alter the natural development of morphological traits such as body and limb form (Bolker & Hill, 2000; Young et al., 2008). For example, hatchery-reared Atlantic salmon (Blanchet et al., 2008) develops deeper body profiles and altered fin length than wild conspecifics, which can reduce their swimming performance. Similarly, Diaz-Gil et al. (2020) demonstrated that sea bream (*Sparus aurata*) juveniles reared under controlled conditions without the presence of predators, developed thinner bodies and shorter caudal regions than individuals exposed to predatory cues. The fitness implications of such morphological changes are difficult to predict in the wild but are likely to be adaptive affecting swimming efficiency (Pakkasmaa & Piironen, 2001), feeding ability (Adams et al., 2003) and predator avoidance (Drinan et al., 2012).

Furthermore, differences in colours, spination, carapace and claw shape between hatchery and wild individuals occur for an array of crustacean species (Davis et al., 2005; Young et al., 2008; Parkes et al., 2011; Daly et al., 2013), affecting their ability to defend against predators, to compete for resources or to access to specific foods. For example, for species whose colouration is background- or substrate-dependent, the colour of the artificial substrate may lead to non-natural pigmentation. When released, these off-colour individuals may be conspicuous and attract predators leading to higher mortality rates (Davis et al., 2005; Young et al., 2008). While there are some general concerns and hypotheses about morphological maladaptation in released animals, specific examples and case studies are not abundant. In a study conducted by Stringwell et al. (2014), Atlantic salmons raised in hatcheries exhibited increased crypticity and a significantly lower occurrence of fin erosion and asymmetry compared to captive control counterparts, just 20 days after being released. These observed morphological variations during the post-release period are likely attributed to phenotypic plasticity and the non-random, selective mortality of maladapted individuals. The fact that erosion decreased with time in the wild, but increased in the hatchery, probably reflects some regeneration under natural conditions and is also consistent with selection against maladapted phenotypes. Further research is needed to understand the long-term effects of morphological maladaptation in released animals.

Growth anomalies

Typically, hatchery-reared animals and their wild counterparts can exhibit several significant growth differences as a result of both genetic and environmental factors (Vøllestad et al., 2004; Lorenzen et al., 2012). Animals held in captivity often come from a limited genetic pool, leading to reduced genetic diversity compared to wild populations. This genetic homogeneity can result in reduced growth potential and adaptability to hatchery conditions (Taniguchi, 2003). In addition, wild animals experience a wide range of environmental challenges including locating food, avoiding predators, and adapting to fluctuating temperatures, which are attenuated under captive conditions, and which impact on their growth patterns (Ut et al., 2007). For instance, large body size is predicted to be beneficial in feeding competition in hatcheries where food is delivered in highly predictable locations, but not necessarily in more unpredictable, food-limited habitats where the monopolisation of resources and high food intake is not possible (Niva & Jokela, 2000; Vøllestad & Quinn, 2003). Höjesjö et al. (2004) demonstrated that the relative competitive ability of dominant and subordinate individuals can change in response to habitat complexity per se. The authors

showed that habitat complexity decreases the growth rate of dominant individuals in relation to subordinates, but that in structurally simple habitats, the pattern is reversed. Nutritional factor can also play a significant role in shaping differences in growth: wild animals have a more diverse and natural diet, while hatchery reared animals typically fed formulated diets (Näslund, 2021). In addition, hatchery-reared animals can experience higher stress levels due to confinement and handling, which may alter their growth rates (Herron et al., 2023). In particular, the crowded conditions in hatcheries can increase the risk of disease outbreaks, which may further impact growth (Yang et al., 2019). All these aspects are extremely important to guarantee a proper growth of reared animals and to reduce growth performance differences with wild conspecifics which could have a negative impact on natural populations and ecosystems. For instance, it has been observed that the release of growth-enhanced Atlantic salmons, displaying changes in several phenotypic traits such as habitat use, morphology and excretion rate, had a significant impact on the invertebrate community and key stream ecosystem functions such as primary production and leaf-litter decomposition (Cucherousset et al., 2021).

Phenotypic homogeneity

Phenotypic variation is widely recognised as a contributing factor to population persistence; multiple phenotypes (polyphenism) expressed within a population allow for adaptation to changing environmental conditions (Kussell & Leibler, 2005). However, when organisms are kept in uniform and unchallenging environments in captivity, they may experience rapid losses in both genetic diversity and phenotypic variation (Mathews et al., 2005; Jones, 2017). Increasing evidence suggests that reduced genetic diversity and phenotypic variation of individuals born in captivity and released into the wild may induce lower fitness than their wild-born counterparts and that these fitness declines can occur after only a few generations in captivity (Snyder et al., 1996; Frankham, 2008; Christie et al., 2012). Several studies, mainly conducted on terrestrial species, have demonstrated that changes in selective pressure, and loss of phenotypic variation (leading to phenotypic homogeneity) in captive populations, has been attributed to poor reintroduction success (Snyder et al., 1996; Fischer & Lindenmayer, 2000; O'Regan & Kitchener, 2005). Moreover, the degree of phenotypic homogeneity may increase with each captive generation, leading to phenotypes vastly removed from the wild phenotype (Wisely et al., 2008; McPhee 2012). Thus, it is apparent that loss of phenotypic variation may have profound consequences, but to date only a few studies have

attempted to investigate why loss of phenotypic variation occurs in captivity and which impact can have on natural populations.

Mitigating the effects of captive environment through phenotypic plasticity

Hatchery-induced deficiencies imply that a given trait is plastic and thus is capable of change to a more natural state if exposed to natural environmental cues (Daly et al., 2020). While replicating the full complexity of the natural environment in rearing facilities is impossible, identifying practical and economically feasible modifications in the artificial environment can efficiently promote development toward a more wild-like phenotype. Laboratory and field experiments designed to identify and mitigate differences through conditioning may improve survivorship and increase the likelihood of successful releases (Olla & Davis, 1998; Davis et al. 2005; Le Vay et al., 2007, Belgrad et al., 2021). In the following paragraphs we suggest cost-effective methods to incorporate environmental cues into rearing that may provide tangible benefits. For example, increased flow in the rearing environment can stimulate physical exercise, influencing post-release traits such as stamina, behaviour, and cognition (Gomez-Pinilla & Hillman, 2013; Franssen et al., 2021). Variability in food supply may affect metabolic and morphological traits, post-release growth, and migration willingness (Garlock et al., 2014; Persson et al., 2018). Environmental enrichment techniques can improve the welfare of animals involved in reintroduction programs while simultaneously enhancing success rates by addressing and mitigating various behavioural, morphological and physical alterations (Shepherdson, 2004; Reading et al., 2013; Arechavala-Lopez et al., 2020).

Determining the optimal duration of conditioning is also crucial for achieving the desired outcomes without causing undue stress or negative effects on the reared organisms. This duration will vary with species-specific requirements and depend on the targeted phenotypic traits. For instance, the enhancement of sheltering behaviour could occur after short-term exposure (days; Carere et al., 2015), while morphological conditioning may operate over extended temporal scales due to involved physiological mechanisms (such as carotenoid assimilation and muscle development; Miner et al., 2005). Furthermore, taking into account that young animals often display greater plasticity than adults (Liang et al., 2020), is also crucial to identify the most suitable ontogenetic period for a proper implementation of such methodologies. It is well known that a lack of complexity in the early developmental environment of an individual negatively impacts its neural

development, with wide ranging implications for adult behaviour and survival (Strand et al., 2010; Salvanes et al., 2013).

Therefore, for effective planning of conditioning strategies it is important to (i) identify the phenotypes in reared organisms that significantly differ from the wild population and that exhibit ample plasticity, (ii) recognize the environmental stimuli inducing such differences, and (iii) understand how to manipulate the rearing environment – considering typology, timing, and duration of stimuli exposure - to induce the desired traits.

Behavioural conditioning

Within the context of stock enhancement, the short-term nature of behavioural changes is both a cause for concern and a mechanism for the alleviation of that concern. As previously seen, the hatchery environment may induce behaviours that are maladaptive in the natural environment. However, this short-term plasticity in behaviour means that individuals can alter their behaviours after release or that conditioning pre-release may induce such behaviours that continue for a short time after release (Daly et al., 2020; Crates et al., 2022). Considering that initial response of animals to a novel environment is often behavioural, encouraging behavioural plasticity and the development of a wild-like behavioural repertoire presents valid opportunities for conservation programs (Braitwhite & Salvanes, 2005; Maculay et al., 2020). For example, several studies highlight the importance of providing environmental enrichment in the early rearing environment to shape individual behavioural patterns, induce behavioural flexibility and facilitate the emergence of learning capabilities (Dickel et al., 2000; Braithwaite & Salvanes, 2005; Salvanes et al., 2007). Nevertheless, unsuitable enrichment can result in injury, stress, disturbances and increased aggression, therefore degrading fish welfare and impairing the success of conservation efforts (Naslund & Johnsson, 2016). In the following paragraphs, we explore how conditioning and training interventions can address certain behavioural deficiencies of concern for reintroduction activities, thus helping the adverse effects of captivity.

Enhancing predator avoidance skills

Predator aversion can be the difference between survival and rapid mortality, particularly in the first moments after release, so anti-predator conditioning is often essential (van der Meeren et al., 2005; Shier & Owings, 2007). Experimental protocols typically follow two different approaches: (1)

classical conditioning and (2) direct/indirect exposure to predation cues. Conditioning studies use chemical alarm cues from injured conspecifics which is associated to a predator cue. On the other hand, exposure experiment can be conducted using either direct predator-prey interaction or no-contact predator exposure, assuming an innate response. As regard the conditioning approach, laboratory experiments conducted on a variety of aquatic species have yielded contrasting results (Mirza & Chivers, 2000; Vilhunen & Hirvonen, 2003; Wisenden et al., 2004; Jacobsen & Stabell, 2004; Olson et al., 2012). In Arctic charr (*Salvelinus alpinus*), a single conditioning session led to improved antipredation responses, but repeated conditioning (up to four sessions) increased the response further, indicating no habituation effects over these sessions (Vilhunen & Hirvonen, 2003). In contrast, effects of conditioning disappeared already after the second session in chinook salmon (*Oncorhynchus tshawytscha*; Berejikian et al., 2003), indicating that it may be hard to generalize effects across species, even if they belong to the same family. However, only a few studies have tested post-release performance after predator odour conditioning, with less than satisfying results (Hawkins et al., 2007). Studies on direct predator exposure showed highly variable results as well. For instance, the maladapted behaviour of increased daytime activity of captive spiny lobsters (*Jasus edwardsii)* is reduced with the presence of a predator; yet individuals display normal sheltering behaviour once released in the wild regardless of experience with predators (Oliver et al., 2006) and have similar survival rates (Mills et al., 2004). On the other hand, hatchery-cultured juvenile red king crabs (*Paralithodes camtschaticus*) increase crypsis with prior predator exposure (Daly et al., 2012) and individuals without predator exposure experience high mortality in the first days after release (Long et al., 2018). No-contact visual/chemical predator exposure experiments are also variable in their results, with some studies showing modified antipredation behaviours and improved survival in laboratory predation trials, in both fish and gastropods (Kellison et al., 2000; Delgado et al., 2002). One potential explanation for the conflicting outcomes of predator conditioning and training may stem from the realization that avoiding a predator often necessitates not only the recognition of threat but also the animal's capacity to respond flexibly, displaying a propensity to learn and adapt to new situations. While some species possess an innate ability to recognize predators regardless of species or training, other may exhibit responsiveness influenced by species-specific and ontogenetic factors (Daly et al., 2012). These factors, influenced by the subject's developmental stage and the predator species involved, can impact anti-predator responses, and thus warrant further research attention.

Promoting cognitive flexibility and spatial adaptation

Increased environmental complexity, in the form of added physical structures to captive settings (environmental enrichment techniques), is typically intended to enhance the cognitive stimulation of captive animals, simultaneously improving their spatial learning abilities (Salvanes et al., 2013; Arechavala-Lopez et al., 2020) and is also often applied to reduce distress and its negative consequences on performance and welfare (Naslund & Johnsson, 2016; Zhang et al., 2019). Improved sensory stimulation and cognition are commonly expected effects, particularly in animals where positive enrichment-effects on brain size and plasticity have been demonstrated experimentally (e.g., Kihslinger & Nevitt, 2006; Salvanes et al., 2013). Arechavala et al. (2020) found that seabream (*Sparus aurata*) reared under enriched conditions showed increased dopaminergic activity in telencephalon and increased serotonergic activity in cerebellum, leading to an overall enhancement in their exploratory behaviour, spatial orientation and learning capability compared to seabream reared in not enriched conditions. Positive enrichment effects on learning and memory have also been detected in common cuttlefish (*Sepia officinalis*), with effects being dependent on exposure-time (Dickel et al., 2000). Space use ability (e.g. sheltering behaviour) is another aspect of cognition which can be enhanced through environmental enrichment techniques. For example, in territorial species higher sheltering tendency may be adaptive soon after release in the presence of natural predators and has been shown to increase after exposure to structurally enriched rearing environments in both fish (Salvanes & Braithwaite, 2005; Roberts et al., 2011; Naslund et al., 2013; Zhang et al., 2019) and crustaceans (van der Meeren, 2001; Carere et al., 2015), but there are indications that effects depend on ontogenetic stage (at least in fish; Rosengren et al., 2017). Thus, to achieve an improvement in cognition and related behaviours of reared animals it is essential to consider species-specific requirements, such as the timing of exposure, the type of enrichment and their possible interactions with other environmental factors. While a few studies revealed potential increases in survival rates after release or suggestive indications of such effects following pre-release exposure to physical structures (Kawabata et al., 2011; D'Anna et al., 2012; Hyvarinen & Rodewald, 2013**;** Roberts et al., 2014), many other studies have not highlighted similar effects, including studies on some species where positive behavioural or cognitive effects have been detected (Brockmark et al., 2007; Fast et al., 2008; Rosengren et al., 2017; Solas et al., 2019).

Improving morphological development and growth

Changes in morphology and growth observed in animals raised in captivity can result from phenotypic plasticity in response to the altered conditions of captive environment (Milner et al., 2005; Garduño-Paz et al., 2010). Researchers can utilize this plasticity to prevent the development of morphological maladaptation and growth alterations. This can be achieved by incorporating ecologically relevant environmental stimuli into the rearing environment and implementing comprehensive procedures that consider the biological needs of the species, covering various aspects of animal care and management. Such an approach promotes adaptive responses in the organisms, thereby preventing the development of traits that might be maladaptive in their natural environment. Below are reported some examples that show how the introduction of specific environmental stimuli and/or the use of particular rearing techniques are essential for the proper development of morphological traits and for the optimization of growth performances.

Morphological modifications

As previously stated, by introducing ecologically relevant environmental stimuli in the rearing environment it is possible to induce morphological differentiation through phenotypic plasticity, probably generating phenotypes more adapted for the life in the wild. For example, Garduño-Paz et al. (2010) demonstrated that fishes (*Gasterosteus aculeatus)* reared in enriched aquariums (with gravel, large stones with interstitial spaces, and artificial macrophytes) exhibited a more fusiform body and smaller heads which could be favourable traits for movement in complex environments. Moreover, exposure to natural substrate or coloured tank backgrounds has been shown to enhance adaptive colouration (i.e., background-matching) in crabs, cuttlefish, and flatfish (Davis et al., 2005; Parkes et al., 2011; Yasumuro & Ikeda, 2011), which is an essential characteristic to avoid predators. Specifically, crabs and lobsters are able to display colour modifications with short-term exposure to natural sediment or dark tank backgrounds over several weeks (Tlusty et al., 2009; Parkes et al., 2011). In addition, the introduction of natural substrates or shelled prey items in the rearing environment during early-development promotes muscle differentiation, enhances asymmetrical claw development (Wickins, 1986; Govind, 1989; Goldstein & Tlusty, 2003) and improves overall strength in crustaceans (Smith & Palmer, 1994). However morphological changes can also be induced by sensory stimuli, such as those released by predators. For example, cultured blue crabs (*Calinectes sapidus*) develop longer spines when exposed to chemical and visual cues released from

fish predators which conferred them higher survivorship in both laboratory and field experiments, suggesting that this feature may be one on which to focus large-scale conditioning efforts (Davis et al., 2005; Young et al., 2008). Furthermore, Delagado et al. (2002) observed that conch (*Strombus gigas*) conditioned with predator cues grew slower than the naive conch but developed denser shells, leading to higher survival rate in a subsequent predation experiment. These findings align with those of Belgrad et al. (2021) who noted that juvenile (*Crassostrea virginica*) exposed to predatory cues exhibited increased shell strength and thickness compared to the non-exposed oysters, resulting in enhanced survivorship post-release. However, it's worth noting that the morphological responses may be predator specific and stage dependent (Young et al., 2008; Parkes et al., 2011).

Fostering growth patterns

Accelerated development is seen as a bonus in aquaculture, not only for commercial but also for economic purposes, because it allows faster turnover and thus lowers production costs and risks (Thorpe, 2004). This objective is pursued through various means, such as selective breeding or promoting competitive dominance among individuals with faster growth. Size grading (rearing small and large individuals separately) is commonly used in aquaculture to improve survival, growth, and feeding efficiency (Ahvenharju et al., 2005; Zmora et al., 2005; Daly et al., 2012). An illustrative example is found in the enhanced survival of early benthic phase red king crab (*Paralithodes camtschaticus*) in hatcheries, achieved by segregating large individuals from communal rearing tanks (Daly et al., 2012). Besides size grading, providing proper healthcare is critical for optimizing the growth, development and reproductive performance of reared animals, thereby increasing their chances of survival, especially during reintroduction into the wild. Ensuring enhanced nutrition through well-balanced and nutritious feeds further contributes to the normal growth and reproductive success of reared animals (Samat et al., 2020). Environmental enrichment techniques also play a role in promoting growth. Studies in species like the Atlantic halibut (*Hippoglossus hippoglossus*) and seahorse (*Hippocampus erectus*) demonstrate improved growth with the introduction of different stimuli, such as sand gravel and light-coloured plastic plants (Ottesen et al., 2007; Lin et al., 2009). Another crucial factor that requires careful consideration to ensure adequate growth rates is the size and quality of the space provided in the rearing environment as demonstrated by several studies conducted on lobsters where individuals reared in restricted spaces

grew less and slower than conspecifics held in wider compartments (Aiken & Waddy, 1978; Van Olst and Carlberg, 1979; Manor et al., 2002; Latini et al., 2023).

Stimulating phenotypic diversity

Diversity in trait expression among individuals, even if plastically derived, plays a crucial role in ecosystem biodiversity and is essential for the success of post-release efforts (Watters & Meehan, 2007; Pfennig et al., 2010; Cordero-Rivera, 2017). Organisms facing dynamic environmental conditions can adapt by altering their phenotypes, generating a spectrum of optimal traits suited to various environments (DeWitt et al., 1998) which in turn provides an adaptive advantage, enhancing organismal fitness (De Jong, 2005; Reed et al., 2010). However, captivity, as observed previously, often provides a static 'ideal' environment, lacking the necessary environmental fluctuations to encourage the expression of a diverse range of phenotypes or the occurrence of stochastic phenotype switching (Kussell & Leibler, 2005; Mathews et al., 2005). To improve rearing programs for conservation purposes, therefore it is advisable to enhance rearing methodologies to better reflect the environmental features of the reintroduction environment. This can involve introducing environmental challenges during rearing, such as exposure to the original cause of decline (Fischer & Lindenmayer, 2000), environmental heterogeneity (West-Eberhard, 1989; Maynard et al., 2004), parasitism (Summers et al., 2003) or seasonal changes such as food availability. For instance, incorporating structural enrichment in the rearing environment can increase morphological variability of fish (Saraiva & Pompeu, 2014, 2016), while exposure to parasites may induce polyphenism fostering variation in reproductive traits, such as courtship displays and genital morphology (Summers et al., 2003). However, our understanding of how rearing environments influence phenotypic trait variations is currently limited and additional research is required to determine which are the best rearing methodologies to foster phenotypic variation.

Opportunities and challenges for conservation aquaculture

Successful use of hatchery-reared juveniles to enhance recruitment-limited populations or severely depleted stocks is contingent upon their ability to survive and grow upon release into the wild. This ability can be compromised by standard hatchery rearing protocols which often result in juveniles that exhibit behavioural, morphological as well as physiological, characteristics different from their wild counterparts. Many of these phenotypic expressions may be maladapted to the wild, but the

very plasticity that causes these traits suggests that they are reversible. Through conditioning strategies such as sensory stimulation, prerelease training and exposure to natural features, it is indeed possible to alleviate some hatchery-induced deficiencies, increasing the chances of post-release survival (Olla & Davis, 1989; Brown & Smith, 1998; Kellison et al., 2000; Belgrad et al., 2021). This approach holds the potential to enhance rearing program outcomes, provide insights for developing methodologies that improve welfare conditions, minimize unfavourable phenotypic changes, and contribute to the success of aquaculture conservation programs (Mathews et al., 2005; Smith & Blumstein, 2008; Evans et al., 2014).

However, despite a long history of research in this area, development of appropriate and robust interventions to induce the ecological competence and life-skills needs to be further addressed (Lorenzen et al., 2010). Our examination of studies spanning diverse taxa underscores a significant knowledge gap, particularly concerning invertebrates and non-salmonid fish. The current state of knowledge poses challenges in providing a comprehensive assessment of our ability to enhance the post-release performance of stocked aquatic animals. In our current phase, we are exploring diverse strategies, frequently through species-specific case studies, rather than concentrating on refining or generalizing methodologies. Future research efforts should focus in this direction, contributing to a more systematic and effective approach to enhancing the post-release performance of stocked aquatic animals.

Future directions

Although the average effects of captivity on plasticity and phenotypic development have been extensively documented, few studies have delved into the post-release effects. Admittedly, post-release studies are often challenging and expensive, yet they are also essential for validating inductions based on laboratory results. Indeed, while encouraging outcomes are seen in controlled laboratory conditions, it's crucial to acknowledge that these effects may differ in more intricate and fluctuating natural environments. Therefore, it's essential to assess the effectiveness of such interventions in the post-release period before recommending their broad implementation.

In addition, the vast majority of existing studies have predominantly focused on the average effects of rearing conditions on morphological, behavioural and life-history traits, overlooking variation at the individual level. Those studies ignored that phenotypic variation among and within individuals can be important not only for population performance, but also for community

interactions and ecosystem function (Hughes et al., 2008; Bolnick et al., 2011), with important implications for conservation and management outcomes (Hughes et al., 2019). Thus, understanding how different rearing conditions may influence individual trait variation is critical for the success of conservation programs, and is also a noteworthy consideration for animal welfare (Webster, 1995; Zhang et al., 2019).

Finally, a better understanding of mechanisms for induced plastic responses, the roles of exposure duration and response magnitude on conditioning potential for a broader range of species is needed before such methodologies could be adopted on large-scale aquaculture settings. From these conclusions, a number of relevant questions for future research arose:

• For how long post-release do cultured animals from an altered environment differ compared to standard-reared and wild individuals?
• Can the cultured animals generalize learned responses to situations similar, but not identical, to the conditioning phase?
• How individual phenotypic variation is influenced by training or conditioning interventions? Are specific phenotypes promoted over others when an intervention is applied and, if so, how does the surviving part of the stocked population compare with the phenotype distribution of natural populations and standard-reared groups? To maintain a certain degree of phenotypic variation, do different populations or species require different amounts of conditioning?
• How can successful experimental results be applied in large-scale production?
• How do different varieties of the same type of factor (e.g., different prey species, predator cues, enrichment structures, etc.) affect performance?
• How do different types of interventions interact?

Literature survey

I conducted a literature search on Scopus for the terms (Plasticity OR phenotypic OR phenotype) AND (Aquaculture OR restocking OR “stock enhancement” OR reintroduc*) AND NOT (Microbiology OR disease) in titles, abstracts or keywords. The initial search was conducted on December 2023, yielded 2315 studies and after a refinement with subject area, source, language and keywords and a revision of the abstracts for the exclusion of all studies not referring to marine animals, I reached 808 studies. Following a personal and additional review of the topics discussed in each article, I arrived at a final count of 174 articles that constitute the central focus of this review.

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3. Limited holding space reduces growth and behavioural performance in juvenile European lobsters

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Abstract

Conservation aquaculture offers important tools to secure sustainable animal supplies for human use. However, intensive aquaculture practices are increasingly raising concerns about the welfare conditions of farmed animals, which typically face high mortality rates due to diseases, cannibalism, and aggression. Here we focus on the European lobster (*Homarus gammarus*), an economically and ecologically important decapod species that has been subjected to conservation programs for decades. While standard husbandry procedures typically result into large numbers of juvenile lobsters produced and released, it remains poorly known whether such standard settings alter the development of behavioural and life-history traits of ecological importance for the animals to survive in the wild. Here, we investigate whether the size of the individual holding spaces affects the behavioural traits (exploration, space use, and sheltering) and growth (carapace length, intermoult period, and percentage moult increment) of juvenile lobsters during their early benthic stages. Our results offer solid evidence that rearing lobsters into small holding spaces not only limits and slows down their overall growth, but also compromises the development of ecologically-relevant behaviours that are essentials for these animals to survive in a complex and risky environment. We point the attention toward the benefits of adopting better rearing procedures to improve the welfare conditions of hatchery-cultured lobsters that can, in turn, result into faster growth rates and the development of competent behavioural repertoires of these valuable organisms—likely to improve the effectiveness of conservation programs.

Introduction

As the aquaculture industry continues to grow, public concern over the welfare of farmed animals arises (Macaulay et al., 2022; Browing et al., 2023). The growing importance of welfare issues in aquaculture comes both from ethical considerations and from the perspective of improving standards and quality of aquaculture products for economic and conservation purposes (Conte, 2004; Segner et al., 2019; Carere and Mather, 2019). From an economic perspective, increasing attention to the animal welfare may improve the quality of the aquaculture products and reduce animal losses (Sinclair et al., 2019). Individuals kept under appropriate welfare conditions are less stressed and less susceptible to diseases and therefore they require less medication and treatment, show faster growth rates and ultimately provide a better-quality product than individuals maintained under poor rearing conditions (Fernandes et al., 2021). In addition, consumers are becoming increasingly aware about welfare issues associated with intensive production practices, with growing expectations around the quality and safety of aquaculture products (Bennett et al., 2011). There are also benefits related to conservation aquaculture, focused on management and conservation practices of natural populations (Braithwaite and Salvanes, 2010; Froehlich et al., 2017). These strategies aim to release captive reared organisms to increase the natural or wild biomass of the species concerned (Bell et al., 2005). However, the impact of such releases is irrelevant if the individuals that are produced are maladapted for life in the wild. Hatchery-cultured juveniles often develop inappropriate and abnormal phenotypes such as poor antipredator responses and/or impaired exploratory/foraging abilities (Svasand et al., 1998; van der Meeren, 2005; Daly et al., 2020) consequently leading to poorer survival rates than their wild counterparts (Munro and Bell, 1997). Thus, under a conservation aquaculture perspective, ensuring welfare conditions of hatchery-cultured individuals would guarantee the development of competent behavioural repertoires comparable to wild-grown conspecifics which are essential to thrive in the natural environment (Braithwaite and Salvanes, 2005, 2010; Bardera et al., 2019). However, welfare concerns have been historically vertebrate-centric while invertebrates have been less in the focus of welfare research and regulations (Albalat et al., 2022; Wahltinez et al., 2022). Given the recent evidence of pain perception and stress in aquatic invertebrates (Carere and Mather, 2019; Birch et al., 2021), welfare considerations provide opportunities for advancements. Thus, we advocate the use of these advancements and further investigations in this underrepresented but important field of animal welfare.

Holding space (also known as space allowance) and stocking density are among the most important factors that affect welfare and production of animals in aquaculture (Toni et al., 2019; Saraiva et al., 2022) and they need to be carefully considered to accurately plan rearing protocols for any production activity as well as for the effectiveness of conservation programs. However, while there is an extensive literature about the effects of stocking density on growth performance, physiology and behaviour of communally reared species (e.g. M'balaka et al., 2012; Jia et al., 2022; Espinoza-Ramos et al., 2022), the effects of space allowance are still poorly investigated (e.g. Aiken and Waddy, 1978; Nicholson et al., 2008). Stocking density refers to the amount of biomass (kg) or individuals per unit of space and it is usually used for communal reared species (Ellis et al., 2002), while holding space is generally used to describe the amount of space available to a single individual (Petherich and Philip, 2009). This last factor is especially important for territorial species as in the case of several decapods increasingly reared for commercial and conservation purposes and on which there is a growing attention on welfare conditions (Gherardi et al., 2012). However, if these parameters are not carefully managed on the basis of individuals needs and requirements, both crowding and isolation could lead to increased levels of stress hormones, reduced welfare, aberrant behaviours and impaired growth (Montero et al., 1999; Gornati et al., 2004; Guo et al. 2022; Ojelade et al., 2022). Thus, as recommended by the Farm Animal Welfare Committee it is necessary to provide a sufficient amount of space so that animals would be "able to express their most normal behaviour with minimal pain, stress and fear" (FAWC, 1996).

The European lobster (*Homarus gammarus*) is amongst the most valuable shellfish products in the Mediterranean, and it has been subjected to conservation programs for decades (Ellis et al., 2015; Hinchcliffe et al., 2022). Stage I-III larvae are planktonic and are communally reared in aerated upwelling vessels at low stocking density (~ 20 larvae l^{-1}). When larvae reach the first benthic stage (Stage IV), they are moved to individual rearing units to avoid mortality due to cannibalism and aggression, which require intense labour, substantial costs, and a large footprint in terms of space (Swiney et al., 2012). Recently, thanks to technological advances, post-larval semi-automated rearing systems have been developed to allow a higher productivity while reducing costs, labour and surface footprint (e.g. Aquahive® technology). However, these systems tend to restrict substantially the individual holding space compared to conventional systems with potential collateral effects on the welfare and development of reared animals (Van Olst, 1979; Nicholson et al., 2008). It is therefore crucial to investigate the consequences that such standard, minimalistic raising settings could exert

on the quality, in terms of welfare and ecological competence, of individuals destined to such conservation practices.

In this study, we tested whether and how the holding space affects behavioural responses, typically recognized as an important indicator of health and welfare in cultured animals (Martins et al., 2012, Albalat et al., 2022; Barreto et al., 2022), and growth performances of the European lobster (*H. gammarus*) during its early benthic stages. To do so, we focused on three behavioural traits related to exploration, space use, and sheltering (which are presumably critical factors for survival in the wild; Botero and Atema, 1982; Castro and Cobb, 2005; Mehrtens et al., 2005) and three morphometric proxies of growth during the first three benthic stages of our lobsters, which were reared either in large or in narrow individual space.

Materials and methods

The experiment was carried out at the Ichthyogenic Experimental Marine Centre (CISMAR, Tarquinia - VT, Italy - 42.201851, 11.721749), as part of an ongoing restocking project.

Post-larvae production

During the summer 2020 four ovigerous females were caught by local fishermen from two distinct locations along the Tyrrhenian coast (Fig. S1; Porto Ercole - 42.393183, 11.211192, and Montalto Marina - 42.327235, 11.572900) and transported to CISMAR facilities. Berried females were housed in separated 1500 L culture tanks with a 1.5 water turn-over rate connected to a RAS (Recirculating Aquaculture System) over a four to five months period, till eggs hatching. Water flowed through a suspended solid removal unit (63µm mesh), a moving bed bio-filter reactor, a foam fractionator and a UV sterilizer before flowing back to the holding tanks. Water temperature was set at 17°C. Salinity, dissolved oxygen, pH and nitrogen compounds (TAN; NO_2^-; NO_3^-) were monitored daily and kept within their optimal range (~38 ‰; ~9.5 mg/L; ~8.4; <1.0 mg/L; <0.4 mg/L; <20 mg/L). Newly hatched larvae were collected every morning, counted and housed in 200 L upwelling vessel with heavy aeration over the entire planktonic phase (stage I-III). The upwellers were connected to the same RAS, and thus were provided with the same water (17°C) of the culture tanks. Water flow was set at 300 L/h to guarantee a 1.5 water turn-over rate. Larvae were stocked at 20 larvae l^{-1} (Hughes et al., 1974; Beard et al., 1985; Bell et al., 2005) and fed *ad libitum* twice a day with a mix of frozen *Artemia* sp., *Mysis* sp. and krill (*Euphasiidae* sp.).

Experimental procedure

Once reached the benthic phase (stage IV), approximately two weeks from hatching, a total of 64 post larvae were equally and randomly divided between two treatments characterized by holding spaces of different shape and size reflecting the dimensions provided by conventional and new-generation post-larval rearing systems. The wide treatment (e.g. conventional system) was composed by square-shaped containers 8 cm per side, 3 cm deep with a 64 cm^2 surface area (Fig. 1a), while the narrow treatment (e.g. new-generation system), characterised by narrower individual spaces, was composed by trapezoid containers 2 cm depth with a 6 cm^2 surface area (Fig. 1b). Each treatment was divided in two batches consisting of identical floating grids, each one located in different 1500L tanks in order to avoid a tank effect. Individual compartments of both treatments were made up of solid plastic walls with no cover and a 1 mm^2 perforated bottom to ensure water exchange. The juveniles were deprived of any form of enrichment (e.g. substrate, shelter, predator cues etc.). Uneaten food and faeces were syphoned out daily. Lobsters were allowed to consume their exuviae. All juveniles were subjected to these treatments until they reached stage VI (approximately 8 weeks from hatching) and they were fed daily with a mix of frozen *Artemia* sp., *Mysis* sp. and frozen krill (*Euphasiidae* sp.) administered *ad-libitum*. All the 4 tanks (2 for each treatment) were provided with controlled sea water from the same RAS described above. The lighting schedule was maintained on a semi-natural dark:light cycle (8:30- 17:30) with lamps (36W) controlled by a timer and complemented with the natural light spreading through the large windows of the laboratory facility.

Behavioural tests and morphometrics

Juveniles were tested in a novel environment to analyse their exploratory (e.g. horizontal locomotion), space-use (e.g. time spent in contact with the sides of the arena) and sheltering (e.g. time spent inside the shelter) behaviours. Each individual was tested during each inter moult periods at stage IV, V and VI respectively. In addition, photos of each juvenile were taken with a camera (Fujifilm xp140) before the start of each test. The test tank (Fig. 2) was provided with the same sea water taken from the RAS already described ($\simeq$ 17°C) and was composed of 4 visually isolated rectangular compartments of 21,5 cm X 11 cm and 11 cm height characterised by the presence of gravel substrate ($\simeq$ 2 mm) and shelter (PVC pipe) in order to simulate a more natural environment.

Each juvenile was moved from its individual container and gently released inside a PVC cylinder located in the left corner of each test compartment. After one minute, the PVC cylinders were simultaneously removed, and juveniles were able to explore the novel environment for a total of 5 min. Between each group of tested juveniles, the test tank was emptied, cleaned, and refilled with the sea water taken from the RAS in order to remove chemical cues from previously tested lobsters. At the end of the test, juveniles were put back into their respective compartments. The test tank was located in the same room with the same light conditions of the rearing tanks.

Behavioural traits were recorded with a GoPro Full HD camera (48 frame per second), located above the test tank (Fig. 2). Behavioural analyses were performed with the software Boris 5.1.3 (Friard and Gamba, 2016), blind to the treatment, to obtain duration and frequencies of all the considered behaviours. In addition, from each tested juvenile the following life-history parameters were recorded: CL (Carapace Length, mm), IP (Inter-moult Period, days), and the PMI (Percentage Moult Increment, %). Carapace measurements were obtained from the photos using the software ImageJ. PMI was calculated by using the following formula (Castro, 1992, González-Gurriarán et al., 1995, 1998): PMI = ((post-ecdysis CL - pre-ecdysis CL)/pre-ecdysis CL)*100.

Data analysis

Overall, 64 juvenile lobsters were assayed, giving a total of 183 observations; three individuals did not complete the study due to early mortality. The individuals that underwent all the three replicates of the behavioural test were balanced across treatments (Wide $N = 31$; Narrow $N = 30$). Data analysis was performed with *RStudio* v. 4.2.1 (*R* Core Team, 2016), using the packages *lmerTest* and *Emmeans* (Kuznetsova et al., 2017; Lenth, 2018). We used default families in the mixed models and verified the normality of the weighted residuals by using quantile-quantile plots and lines crossing the first and third quartiles, although linear mixed models are robust to violations in distributional assumptions (Schielzeth et al., 2020). The significance level was set at $\alpha < 0.05$. We wanted to test whether juveniles reared in wide and narrow housing conditions differed respectively in their behaviour (horizontal locomotion, time spent in contact with the walls, and time spent inside the shelter) and life-history traits (body size, intermoult period, and percentage moult increment). Accordingly, we fitted generalized linear mixed-effects models with exploration (i.e. horizontal locomotion, in s), space use (i.e. time spent in contact with the walls, in s), sheltering (i.e. time spent inside the shelter, in s), body size (i.e. carapace length, in mm), intermoult period (i.e. time between two consecutive moults, in days), and percentage moult increment (i.e. mean increase in carapace

length per moult, in %) as the dependent variables. Individual identities (random intercepts) were included in the random structure of each model to account for repeated measures, while the fixed effects were holding space (size of the housing enclosures), stage (moult IV, V and VI), their interaction (holding space x stage), batch (two identical floating grids for each treatment), test duration (possible divergences from the 5 min.) and mothers (four levels). Accordingly, a significant holding space x stage interaction in the fixed-factor structure of the models would indicate that individual holding space had different effects on the behaviour and/or on the life-history of lobsters over their development. We ran pairwise comparisons with the conservative Bonferroni method for significant predictors and accounted for the variation explained by other predictors.

Results

Generally, the space availability had a strong effect on lobsters' growth and behaviour during the three developmental stages (Figs. 3 and 4, Tab. 1 and 2, Tab. S1). As regard behavioural traits, holding space significantly affected activity (horizontal locomotion) and space-use (time spent in contact with the walls), and a similar trend was also observed on sheltering behaviour, although it failed to reach statistical significance (Tab. 1). Overall, juveniles reared in wider holding spaces spent more time moving (Fig. 3a) and hiding in the refuge (Fig. 3b) and less time in contact with the walls of the arena (Fig. 3c) than conspecifics reared in narrower spaces. On the other hand (Fig. 3d,e), juveniles reared in wider spaces slightly decreased their time spent moving as well as time spent in contact with the walls with increasing age (i.e. stage), differently from individuals reared in narrower spaces in which activity and time spent in contact with the walls increased during subsequent developmental stages; in any case juveniles belonging to the wide treatments tourn out to be, at any developmental stage, more active than the conspecifics belonging to the narrow treatment. Sheltering behaviour also showed significant differences between the two groups, but only as regards the interaction between holding space and age (Fig. 3f). In fact, during their three first benthic stages individuals reared in wider spaces spent longer time inside the shelter than conspecifics confined to narrow compartments, which tended to use the shelter less with increasing age. These last results indicates that the effects of holding space on behaviour are not consistent over lobster's development.

With respect to the life-history traits measured, the amount of individual space provided to the lobsters had, on average, significant effects on both carapace length and intermoult period. Specifically, juveniles held in wider compartments were on average bigger (Fig. 4a) and grew faster

(i.e. shorter intermoult periods; Fig. 4b) than conspecifics reared in narrower enclosures. However, these average differences in body size and intermoult period between juveniles belonging to the two treatments were not constant and became more pronounced with increasing age (Fig. 4c,d). In addition, the interaction between holding space and age showed significant effects also on the percentage moult increment (PMI) with individuals reared in wide compartments scoring higher PMI between two consecutive intermoult periods (stages IV-V and V-VI) than juveniles held in narrow spaces (Fig. 4e). Therefore, also in this case the effects of holding space on all the life-history traits considered in this study are not consistent over lobsters development.

Discussion

Our results provide a clear perspective on the effects of holding space on the development and welfare of hatchery-reared European lobsters. We report novel experimental evidence that consistent differences on the average behavioural and life-history traits of juvenile lobsters are associated with different individual compartment sizes. Specifically, juveniles reared individually in restricted spaces moved on average less, spent less time hiding in the refuge and more time in contact with the sides of the arena than conspecifics reared in larger compartments. In addition, juveniles showed behavioural differences during their development that were explained by the variation in the conditions in which they were reared. For example, lobsters reared in restricted compartments increased their locomotory activity and the time spent in contact with the walls while decreased their shelter use over developmental stages, contrary to individuals reared in larger holding spaces. These results indicate that providing a sufficient amount of space to individually hatchery reared lobsters may positively affect the development of ecologically relevant behaviours, which are essentials to cope with the challenges provided by the natural environment. Finally, results on morphometric measurements and intermoult period revealed that juveniles reared in smaller compartments grew, on average, less and slower than conspecifics reared in larger ones, which is consistent with previous studies. However, as showed by the results, the differences on growth performances between juveniles belonging to the two treatments were not equal for each developmental stage but tended to become more pronounced with increasing age. Thus, finding a holding cell size that does not inhibit juvenile European lobsters' growth allows researchers and aquaculturist to balance production goals and methods to produce adequate numbers of healthy juveniles. Overall, our evidence suggests that environmental factors, such as space availability, play

a pivotal role in shaping differences in behavioural and life-history traits averagely expressed in a marine crustacean species, at least in the short term.

While there is an extensive literature about the effects of stocking density and housing conditions on the behaviour of several communally reared species (e.g. Polverino et al., 2015; Maierdiyali et al., 2020; Jia et al., 2022; Espinoza-Ramos, 2022; Saraiva, 2022), almost nothing is known about the effect of holding space on behaviour of individually reared animals, especially regarding aquaculture species, which often requires communally rearing techniques (Tennessen, 1989; Pearce and Paterson, 1993). As concern lobsters, to date, several studies have investigated the effects of "training" interventions such as sensory stimulation (van der Meeren, 1993), pre-release conditioning (van der Meeren, 2001; Agnalt et al., 2017), and exposure to natural features within the hatchery environments (Wickins and Barry, 1996; Carere et al., 2015) on ecologically-relevant behavioural traits. Yet, to the best of our knowledge, this is the first study to have tested for the effects of holding space on the behavioural performances of juvenile lobsters. As evidenced by the results, rearing juveniles in a very confined space, on average, may impair their locomotory activity as well as their shelter and space use behaviour. While a juvenile lobster reared in a wide space has the possibility to explore the sorrounding environment changing freely its position and direction, conspecifics reared in narrower spaces might get accustomed to stay in costant contact with the lateral surfaces, which also offer a sense of security, to the detriment of their exploratory ability. In addition, juveniles held in narrow compartments spent, on average, less time hiding in the shelter than conspecifics of the wide treatment, indicating that restricted holding space may impair their shelter use ability, which is considered one of the main requirements for the survival in the natural environment, especially within the first hours after release (Cobb, 1971; van der Meeren, 2005). Under this perspective, restricted space may impair their spatial awareness and cognitive ability with major consequences on locomotory activity, space use, and sheltering behaviour (Arechavala-Lopez et al., 2020). Whether or not restricted space may cause chronic physiological stress as it was shown upon changes in water conditions (e.g. Chang., 2005; Freire et al., 2020) warrants further investigation. These results demonstrate that behavioural and coping abilities of reared lobsters are affected not only by the structural, social, or sensorial complexity of the rearing environment but also by the available space provided.

In addition, it is also important to consider the behavioural ontogenetic shifts experienced by juvenile lobsters during the early benthic phase in order to implement procedures to increase their competence and welfare state, and to detect specific temporal windows in which they might

be more fit to be released. According to Castro and Cobb (2005), juveniles of the American lobster (*Homarus americanus*) are more active during the first benthic stage (stage IV) and become less active and more prone to benthic behaviour (e.g. burrowing, sheltering) later in their development, indicating the presence of a defined shift in behavioural patterns between early and late stage IV, where swimming behaviour changes to sheltering (Botero and Atema, 1982; Cobb et al., 1989). Considering that predation is the first and most risky challenge after release, (van der Meeren, 2001; Oliver et al., 2008; Daly et al., 2013) reducing roaming time and locomotory activity in favour of shelter occupancy could be beneficial for hatchery-reared lobsters and may increase their survival chance in the sea (van der Meeren, 2001). In this study, we observed that rearing juveniles during their early benthic phase in restricted space may alter their behavioural development with potential negative consequences on the success of conservation activities. In particular, we found that juveniles raised in restricted compartments increase their locomotory activity and the time spent in contact with the walls while decrease their shelter use over developmental stages, contrary to individuals reared in wide holding spaces that instead reflect the ontogenetic behavioural changes of wild-grown conspecifics (Cobb et al., 1989). Thus, although we have no detailed knowledge of the behaviour of juvenile lobsters in natural conditions, providing enough individual space in the rearing environment is likely relevant for the development of of adequate behavioural competencies of hatchery-reared lobsters.

On the other hand, the effects of holding space on growth performances has already been partially investigated. Templeman (1948) and later Van Olst and Carlberg (1979) stated that in the American lobster restricted space becomes soon a limiting factor to growth by both decreasing increment gain per moult and by protracting the intermoult period. However, Hughes (pers. comm. in Aiken & Waddy, 1978) was the first one to describe the relation between minimum space requirements and total length in *Homarus americanus*, estimating that for unrestricted growth it's necessary to provide an area major or equal to 3 times body length squared $(3xTL)^2$.

Aiken & Waddy (1978) used Wilder's (1953) equation (CL = 0.344 TL – 0.13) to convert Huge's space relationship to ($8.7\ CL^2$) or approximately $75(CL^2)$. This relationship can be expressed as:

$$a = 75\ (CL)^2$$

where a is the minimum amount of space required at a specific CL to avoid growth potential limitations, while 75 indicates the space factor number obtained by the conversion of the previous Huge's space relationship. Our results are in line with the studies carried out by Templemann and

Van Olst, with juveniles reared in narrow spaces that grew less and slower than conspecifics reared into larger enclosures but do not agree with the formula proposed by Aiken and Waddy. As showed by the PMI results, individuals reared in restricted compartments decrease their average increment in size gained per moult, moving from 18% of increment gained at stage V to 15% at stage VI while juveniles raised in wide compartments increase that percentage, moving from 14% of average increment at stage V to 22% at stage VI. These results indicate that the size of the narrow compartment considered in this experiment becomes a limiting factor to growth starting from stage V, while the space provided in the wide compartment is large enough to not alter the growth performances of juvenile lobsters, at least until stage VI. If we consider the averaging CL size of lobsters at stage VI (12 mm) and the area of wide compartments considered in this experiment (64 cm^2), which is sufficient enough for unrestricted growth, the space factor proposed by Aiken and Waddy should be changed from 75 to 44.5. However, reduced growth to space limitation can be reversed by placing the organism in a larger holding space (Van Olst and Carlberg, 1979; Aiken and Waddy, 1988; Wilber and Wilber, 1989), which suggest that inhibited growth is the result of stress and when stress is removed, growth continues normally.

It is important to consider that the effects of individual space requirements in rearing environments are likely to be species-specific and dependent mainly on the animal life-stage. Lobsters and crayfish need a comparatively large compartment for unrestricted growth (Aiken and Waddy, 1978; Goyert and Avault, 1978; Van Olst and Carlberg, 1979; Manor et al., 2002) if compared with the space requirements of swimming crabs that have a radically different body and therefore may interact with their immediate surroundings in a different manner (Nicholson et al., 2008). In conclusion, we demonstrated that the holding space affects not only the individual growth, but effects carry over to behavioural performances and thus to welfare conditions of the European lobster. In fact, behaviour represents a reaction to the environment as animal perceive it and it has typically been recognized as an important indicator of health and welfare in cultured animals (Huntingford et al., 2006; Martins et al., 2012; Albalat et al., 2022; Barreto et al., 2022). It must be highlighted that the welfare issue entails two distinct but overlapping and complementary facets: the ethical aspect also involving invertebrates, which is increasing in society (Carere and Mather, 2019; Birch et al., 2021) and the attention to correct rearing procedures that may guarantee the development of appropriate behavioural phenotypes, which may be especially important in organisms involved in conservation programs. Thus, based on the results of this experiment, we recommend housing juvenile lobsters intended for conservation purposes with holding space of at

least 44.5 times carapace length squared, or 44.5 $(CL)^2$ so that lobsters would be able to grow normally and develop their most natural behaviour. Since little is known about crustacean stress and welfare and because behavioural plasticity typically correlates with physiological changes, further studies will be necessary to investigate the physiological responses experienced by juveniles reared in confined spaces also in combination with environmental enrichment procedures during ontogeny.

Fig. 1 - The two different treatments given the European lobster juveniles (Homarus gammarus). (a) wide treatment composed by square-shaped individual compartments of 64 cm^2; (b) narrow treatment composed by trapezoid individual compartments of 6 cm^2.

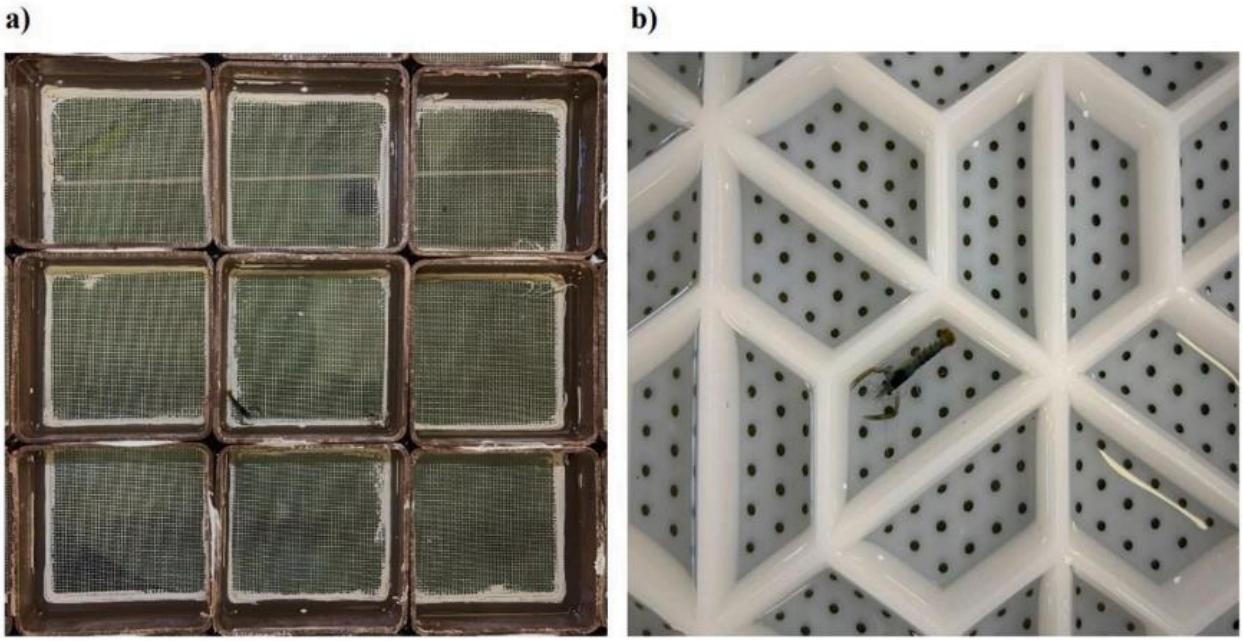
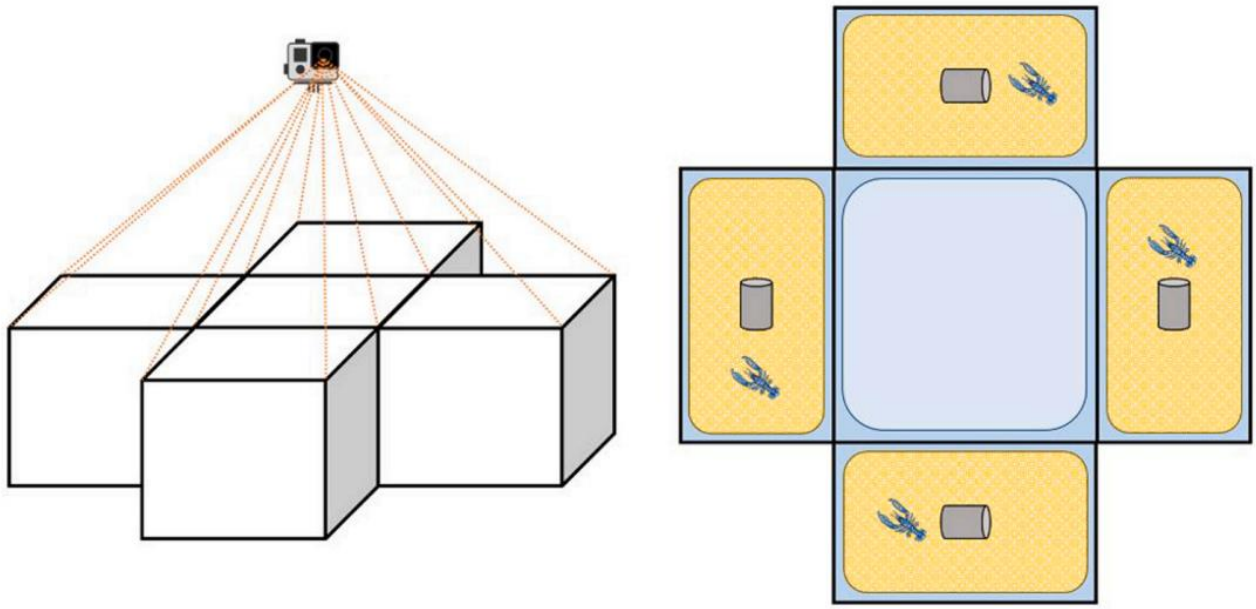
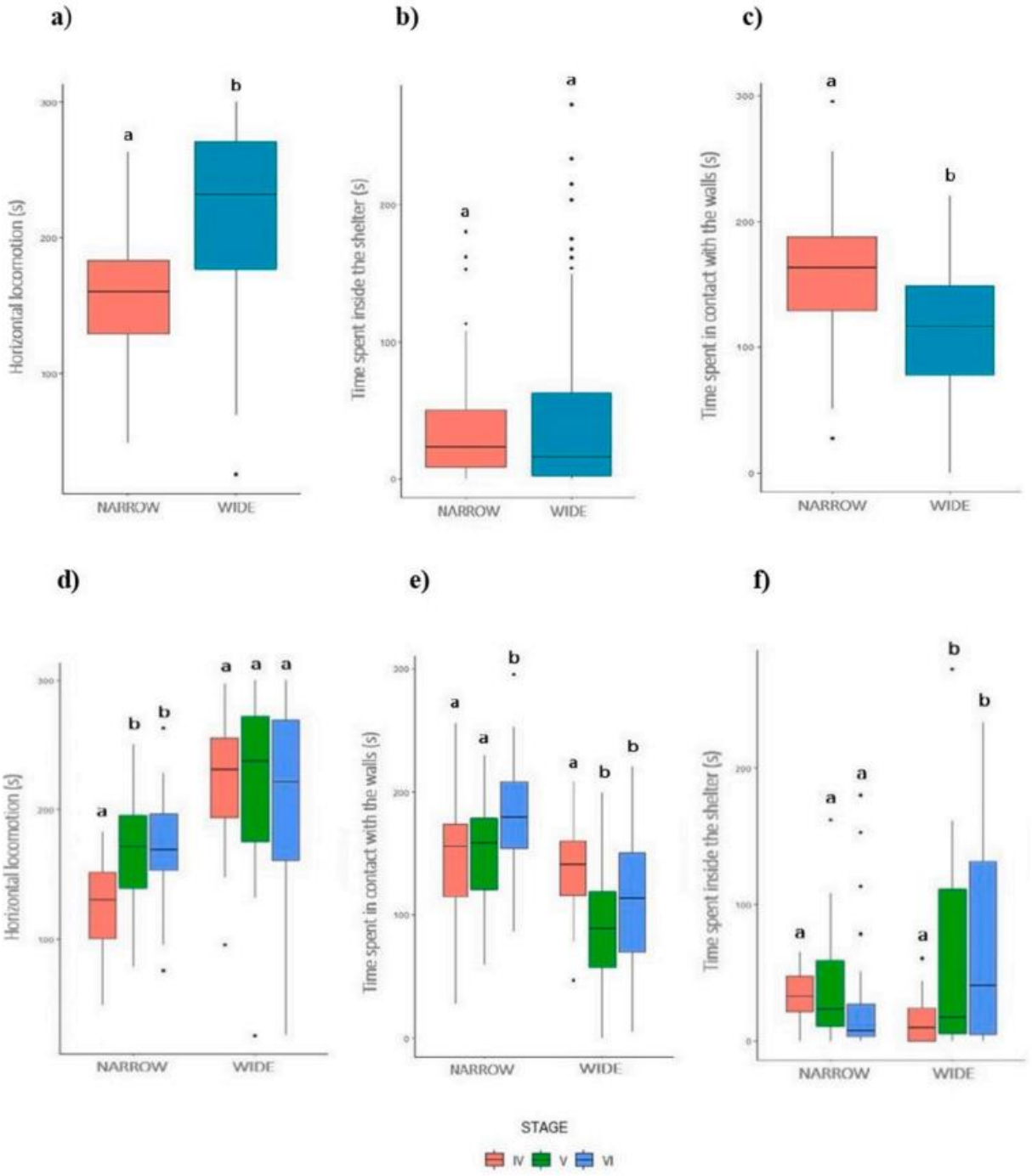


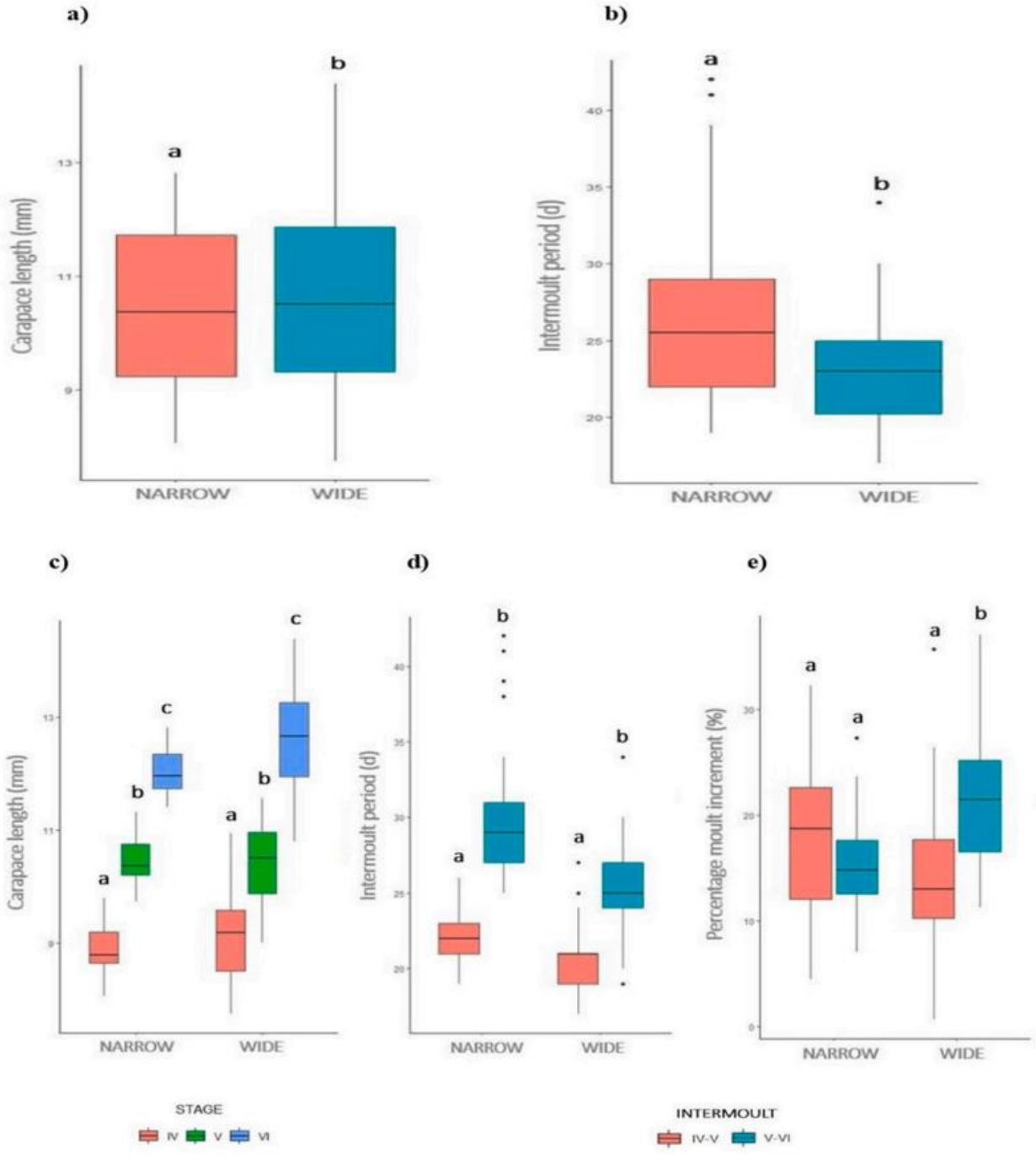
Fig. 2 - Representation of the test tank. The test tank was composed by 4 rectangular compartments provided with substrate and shelter (PVC pipe), a GoPro Full HD camera was located above the test tank to record all the expressed behaviours.



Figs. 3 – **Effects of the holding space and of the interaction between holding space and age on the expressed behaviours.** Average effects of holding space on (a) horizontal locomotion, (b) time spent in the shelter and (c) time spent in contact with the walls, while in the other images are reported the effects of the interaction between holding space and age (e.g. stage) respectively on (d) time spent moving horizontally, (e) time spent in contact with the walls and (f) time spent inside the shelter. Different letters (e.g. 'a' and 'b') indicate significant differences between boxplots. The data are presented as standard box-and-whisker plot with median (horizontal lines), and first and third quartiles (boxes). Whiskers represent minimum and maximum values except for outliers (filled dots).



Figs. 4 - **Effects of the holding space and of the interaction between holding space and age on growth parameters.** Average effects of holding space on (a) carapace length and (b) intermoult period, while in the other images are reported the effects of the interaction between holding space and age (e.g. stage, intermoult) respectively on (c) Carapace Length (CL), (d) Intermoult Period (IP) and (e) Percentage Moult Increment (PMI). Different letters (e.g. 'a' and 'b') indicate significant differences between boxplots. The data are presented as standard box-and-whisker plot with median (horizontal lines), and first and third quartiles (boxes). Whiskers represent minimum and maximum values except for outliers (filled dots).



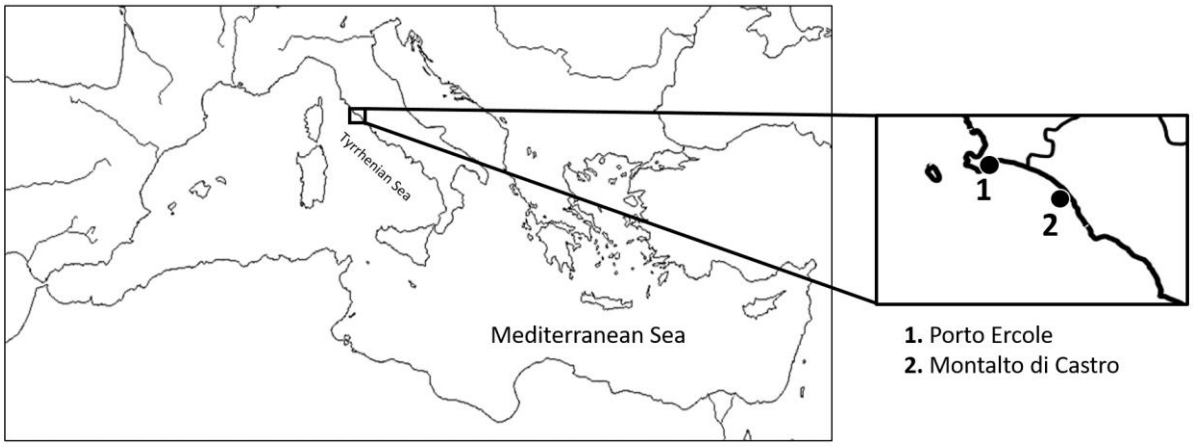
Tab.1 – **Results from the fixed-factor structure of the generalized linear-mixed models.** Holding space (two levels), stage (three repeated measures per individual) batch (two for each treatment), mother (four levels) and the interaction between holding space and stage are included as fixed effects in the models. Significance was $\alpha < 0.05$ and significant results are in bold.

Model				
	Mean Sq.	df	F	P
Horizontal locomotion				
Holding space	64949	1, 61	26.493	**< 0.001**
Stage	8517	2, 121	3.474	**0.034**
Batch	2360	2, 60	0.963	0.388
Mothers	6503	3, 60	2.652	0.057
Test duration	13946	1, 183	5.688	**0.018**
Holding space*Stage	18887	2, 122	7.704	**0.001**
Time spent in contact with the walls				
Holding space	40670	1, 62	21.145	**< 0.001**
Stage	10124	2, 122	5.263	**0.006**
Batch	707	2, 61	0.268	0.694
Mothers	4435	3, 62	2.306	0.085
Test duration	226	1, 183	0.118	0.732
Holding space*Stage	16323	2, 123	8.487	**< 0.001**
Time spent inside the shelter				
Holding space	8073	1, 61	3.606	0.062
Stage	12820	2, 121	5.726	**0.004**
Batch	534	2, 60	0.240	0.787
Mothers	3276	3, 60	1.463	0.234
Test duration	71.4	1, 183	0.032	0.858
Holding space*Stage	16102	2, 121	7.192	**0.001**
Carapace length				
Holding space	1.333	1, 61	8.057	**0.006**
Stage	169.637	2, 122	1024.940	**< 0.001**
Batch	0.006	2, 61	0.035	0.966
Mothers	0.665	3, 61	4.017	**0.011**
Holding space*Stage	1.675	2, 122	10.123	**< 0.001**
Intermoult period				
Holding space	55.470	1, 61	7.327	**0.009**
Stage	1048.830	1, 61	186.102	**< 0.001**
Batch	2.340	2, 61	0.309	0.736
Mothers	3.960	3, 61	0.523	0.668
Holding space*Stage	81.610	1, 61	10.78	**0.001**
Percentage moult increment				
Holding space	0.310	1, 122	0.009	0.924
Stage	170	1, 122	4.960	**0.028**
Batch	56.160	2, 122	1.636	0.199
Mothers	94.860	3, 122	2.763	**0.045**
Holding space*Stage	786.730	1, 122	22.913	**< 0.001**

Tab.2 – Results of the estimated marginal means, accompanied by standard errors and confidence intervals for each dependent variable.

Model	Emmean	SE	df	lower.CL	upper.CL
Horizontal locomotion					
NARROW	158	5.84	70.80	146	170
WIDE	215	5.70	70.50	204	226
HS*STAGE					
NARROW:					
IV	125	9.50	196	106	143
V	176	9.62	196	157	195
VI	173	9.50	196	155	192
WIDE:					
IV	222	9.41	196	203	241
V	217	9.38	196	199	236
VI	206	9.33	196	187	224
Time spent in contact with the walls					
NARROW	160	5.46	70.50	149	171
WIDE	112	5.34	70.20	102	123
HS*STAGE					
NARROW:					
IV	148.20	8.60	196	131.30	165
V	151.40	8.71	196	134.20	169
VI	179.30	8.60	196	162.30	196
WIDE:					
IV	138.20	8.51	196	121.40	155
V	90.80	8.49	196	74.10	108
VI	108.00	8.44	196	91.30	125
Time spent inside the shelter					
NARROW	33.40	5.71	70.90	22.00	44.80
WIDE	48.50	5.58	70.50	37.30	59.60
HS*STAGE					
NARROW:					
IV	34.30	9.16	196	16.21	52.30
V	38.10	9.28	196	19.75	56.30
VI	28.00	9.16	196	9.89	46.00
WIDE:					
IV	14.00	9.07	196	3.88	31.90
V	59.60	9.04	196	41.75	77.40
VI	71.90	9.00	196	54.11	89.60
Carapace length					
NARROW	10.10	0.19	68.90	9.69	10.40
WIDE	10.70	0.09	68.90	10.49	10.90
HS*STAGE					
NARROW:					
IV	8.49	0.20	86	8.10	8.88
V	10.05	0.20	86	9.67	10.44
VI	11.63	0.20	86	11.24	12.02
WIDE:					
IV	9.08	0.11	132	8.86	9.30
V	10.36	0.11	132	10.14	10.58
VI	12.60	0.11	132	12.38	12.81
Intermoult period					
NARROW	26.30	0.50	68.90	25.30	27.30
WIDE	23.40	0.50	68.90	22.40	24.40
HS*STAGE					
NARROW:					
IV-V	22.00	0.61	126	20.80	23.30
V-VI	30.50	0.61	126	29.30	31.70
WIDE:					
IV-V	20.80	0.60	126	19.60	22.00
V-VI	26.00	0.60	126	24.80	27.20
Percentage moult increment					
HS*STAGE					
NARROW:					
V	17.90	1.12	131	15.70	20.10
VI	15.20	1.12	131	13.00	17.40
WIDE:					
V	14.30	1.09	131	12.20	16.50
VI	21.80	1.09	131	19.60	23.90

Fig. S1 - Sampling locations of berried females: (1) Porto Ercole; (2) Montalto di Castro.



Tab. S1 – Individual identities (random intercepts) are included as the random effect of the models to account for repeated measures: intercepts ($V_{between}$) and residuals (V_{within}).

Model

	Estimate	Std.Dev.
Horizontal locomotion		
Vbetween	54.800	7.403
Vwithin	2451.600	49.513
Time spent in contact with the walls		
Vbetween	125.600	11.210
Vwithin	1923.400	43.860
Time spent inside the shelter		
Vbetween	89.550	9.463
Vwithin	2238.870	47.317
Carapace length		
Vbetween	0.181	0.425
Vwithin	0.165	0.407
Intermoult period		
Vbetween	2.671	1.634
Vwithin	7.570	2.751
Percentage moult increment		
Vbetween	<0.001	<0.001
Vwithin	34.330	5.860

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4. Predator cues and environmental enrichment during development drive intraspecific variation in the behaviour and life history of juvenile lobsters

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Abstract

Intraspecific variation in ecologically relevant traits is critical for animal populations to survive in our rapidly changing world. This is especially true for species that suffer from intense harvesting regimes, whereby populations density is often low. Standard hatchery procedures can assist some management and conservation programs by producing large numbers of juveniles to be released into the wild. Yet we know surprisingly little on the impact that such standard, minimalistic settings have on the development of intraspecific variation in important phenotypes of the individuals, including among-individual variation in behavioural (individuality) and life-history traits, and in the plasticity of those traits in response to varying environmental conditions. Here, we fill this gap by testing whether early-life exposure to different environmental conditions alters the development of individuality and plasticity in ecologically relevant behaviours and life-history traits of the European lobster (*Homarus gammarus*)—one the most harvested species in the Mediterranean, which has been subjected to conservation programs for decades. By accessing one of the largest lobsters hatcheries in Italy, we used the progeny of wild-caught females and manipulated—in a full factorial design—the environmental complexity of the individual enclosures (i.e. presence/absence of substrate and/or shelter) and the level of exposure to cues from their natural predators. We repeatedly quantified behaviours (i.e. activity, refuge use, and aggressiveness) and life-history traits (i.e. carapace length and intermoult period) of the individuals throughout their early development, capturing both mean and individual-level variation across treatments. Our results offer solid evidence that effects of standard hatchery settings extend far beyond mean changes in the behaviour and life history of the animals, compromising the development of individual plasticity in those traits that are essentials for populations to survive in the wild—likely reducing the effectiveness of conservation programs.

Introduction

In conservation aquaculture, organisms are typically produced in captivity in large numbers for their reintroduction into the wild, with the aim of restoring depleted populations and enhance wild stocks (Froehlich et al., 2017). However, conventional hatchery-rearing procedures impose substantially different selection pressures on the individuals relative to their natural environment, potentially altering the phenotypic traits that are critical for these organisms to survive in the wild (McDougall et al., 2006; McPhee & McPhee, 2012).

Mounting evidence suggests that changes in the phenotypic composition of a population and in the ability of captive-reared individuals to adjust to changing conditions of their environment can impact the ultimate outcome of wildlife management and conservation programs (McDougall et al., 2006; Mason, 2010; Crates et al., 2023). For instance, phenotypic differences among the individuals are known to be essential for animal populations to survive in our increasingly changing world (Sih et al., 2004; Dall et al., 2012; Polverino et al., 2021). A large portion of such phenotypic diversity within animal groups can be attributed to the effect of different environmental conditions experienced by the individuals (Stamps, 2015), and is quantified as individual-level variation in mean traits and in their plastic adjustments over time and/or across contexts (Dingemanse & Dochtermann, 2013; Snell-Rood, 2013). Nevertheless, while growing evidence suggests that phenotypic variation among individuals can drive evolutionary changes and sustain functional diversity across populations, communities, and ecosystems (Miller & Rudolf, 2011; Des Roches et al., 2018; Raffard et al., 2018), a controversy remains regarding the proximate and ultimate mechanisms of such variation (Polverino et al., 2016).

Early-life conditions experienced by an individual are known to play a key role in shaping the development of its phenotype in adulthood (Monaghan 2008; Frankenhuis & Panchanathan, 2011), and so its capacity to cope with environmental challenges. Yet environmental effects during ontogeny are usually overlooked as the proximate mechanisms behind the emergence of individual differences compared to environmental effects operating across generations (i.e., genetic adaptation; Urszán et al., 2015 and 2018). It is therefore important to understand whether and how environmental conditions can contribute, at least in the short term, to the emergence of phenotypic variation at the individual level. For example, other than improving animal welfare conditions (Zhang et al., 2021), introducing ecological-relevant cues into the hatchery-rearing environment could also benefit conservation programs, by promoting diverse coping strategies and larger investments into phenotypic plasticity (Shepherdson, 1994; Watters & Meehan, 2007; Reading et al., 2013; Daly et

al., 2021). Thus, shedding light on these aspects is critical for managing risks, ensuring the effectiveness of rearing programs, and guarantee high standard of welfare conditions (Webster, 1995; McDougall et al., 2006).

Here, we wanted to test whether and how the early-life exposure to different environmental conditions altered the development of ecologically relevant behaviours and life-history traits of hatchery-reared juvenile European lobsters (*Homarus gammarus*): an ecologically and economically important decapod species, historically targeted for conservation programs. Specifically, 256 lobsters were exposed to different degrees of environmental complexity during their early benthic stages, which included the presence/absence of structural enrichments into their individual rearing enclosures (shelter and substrate) and cues from natural predators. Juveniles were repeatedly assayed during their early development for ecologically relevant behavioural (activity, refuge use, aggression; Réale, 2007) and life-history traits (carapace length and intermoult period; Chang et al., 2012). This approach allowed us to test for treatment effects on both mean and individual-level variation in behaviour and life history (Dingemanse et al., 2010). In accordance with previous studies (Aspaas et al., 2016; Arechavala-Lopez et al., 2020), we hypothesized that standard, minimalistic aquaculture settings would compromise the behavioural repertoire of the animals and alter their growth. Furthermore, we expected that effects of standard hatchery conditions on lobsters' development would extend beyond mean changes in their behaviour and life history, and impair also the capacity of the individuals to adjust their phenotypes to rapid changes of their surrounding environment (e. g. presence of predators). Our analysis sheds light onto the role that early-life environmental stimuli have in shaping mean and individual-level variation in animal phenotypes, offering important insights for science-based improvements of aquaculture practices and conservation programs.

Methods

Study species and experimental animals

We used first-generation progeny of wild-caught European lobsters (*Homarus gammarus*), which were hatched under controlled conditions in the Ichthyogenic Experimental Marine Centre (CISMAR) at the University of Tuscia (Tarquinia, Italy; 42.201851, 11.721749). Four ovigerous females were caught by local fishermen from two distinct locations along the Tyrrhenian coast (Porto Ercole: 42.393183, 11.211192; Montalto Marina: 42.327235, 11.572900) and transported to the CISMAR

facilities. The females were housed separately into 1500L tanks connected to a recirculating aquaculture system, which supplied seawater to the apparatus during the five months until the eggs hatched. The seawater flowed through a suspended solid removal unit (63µm mesh), a bio-filter reactor, a foam fractionator, and a UV sterilizer before entering the holding tanks. Water temperature was set at 17°C, while salinity, dissolved oxygen, pH, and nitrogen (NO_2^- and NO_3^-) were monitored daily and kept within the optimal range for the species (Hinchcliffe et al., 2021).

Eggs were monitored daily and the newly hatched (planktonic) larvae were collected every morning, counted, and housed separately for each female into 200L upwelling, aerated vessels at low stocking density (~20 larvae L^{-1}; Hughes et al., 1974; Bell et al., 2005; Hinchcliffe et al., 2021). The vessels were connected to the same recirculating system described above, so that water conditions experienced by the newborns reflected conditions during their incubation. Larvae were fed *ad libitum* twice a day with a mix of frozen *Artemia* sp., *Mysis* sp., and krill (*Euphasiidae* sp.).

Experimental procedure

Approximately 14 days after birth the larvae reached the benthic stage. Then, 256 juveniles (64 per female) were randomly selected for the experiment and distributed across the experimental treatments, in which the animals were maintained for 24 consecutive weeks (i.e. 168 days). Specifically, four identical floating grids were set up for the experiment, each one placed inside a 1500L tank. Each grid consisted of 64 individual squared-shaped enclosures (8 cm per side and 3 cm deep each), which were made of solid plastic material with a perforated bottom surface (1 mm^2) to ensure the adequate water exchange between the individual enclosures and the housing tank. For each grid, individual enclosures were randomly split across four rearing treatments that differed from each other for the presence/absence of a substrate (calcium carbonate gravel) and/or a shelter (PVC pipe): no substrate and no shelter (control), substrate, shelter, and substrate and shelter (Fig. 1a).

Individuals from two of the floating grids were also exposed to predator cues, for one hour a day, throughout the 24 experimental weeks (predator cues treatment; Fig. 1a). On the contrary, animals housed in the other two grids were never exposed to predators (no predator treatment; Fig. 1a). Each tank containing a grid assigned to the predator treatment hosted five juvenile sea breams (*Sparus aurata,* 10 ± 1 cm in length)—a common predator of small crustaceans (Taieb et al., 2013)—for one hour per day. Individuals in the predator treatment could perceive the presence of their predator via chemical cues (Derby & Sorensen, 2008), but the opaque, perforated material of the

individual enclosures prevented visual interactions between individuals and their predators. After the one hour of exposure, predators were removed and the grids with the experimental lobsters transferred into clean holding tanks. Control grids were also moved into different, clean holding tanks once a day, to balance the procedure across treatments.

Animals were fed daily with a mixture of frozen *Artemia* sp., *Mysis* sp. and frozen krill (*Euphasiidae* sp.) *ad-libitum*. The illumination reflected the light:dark cycle (8:30am-5:30pm) and was diffused through 36W aquarium lamps.

Behavioural assays

The behavioural responses of the juveniles were scored, repeatedly over their ontogeny, across three different behavioural tests: novel environment (NE), predator avoidance (PA), and resident-intruder (RI, Fig. 1b). Behavioural tests were always performed one week after the animals moulted to standardize the procedure, as suggested by Tamm and Cobb (1978). To do so, the days between moults were monitored per each individual throughout the experimental campaign.

The NE and PA tests were performed on each animal consecutively in an experimental arena, in this order, to minimize manipulation and prevent potential predator influence on the initial response of the lobster to the new environment, for a total of three replicates per individual (approximately at 3, 6 and 10 weeks post hatching). Instead, the RI test was repeatedly performed, twice on each individual, within each individual's rearing enclosure.

Behavioural trials were recorded with a GoPro Full HD camera (48 frame per sec). Behavioural videos were scored, blind to the treatment, with the software Boris 5.1.3 (Friard & Gamba, 2016), to obtain latencies, duration, and frequencies of behavioural traits.

Novel environment and predator avoidance test

The experimental arena consisted of two central, squared tanks (21.5 cm per side and 11 cm high; predator areas) surrounded by six rectangular test tanks (21.5 cm long, 11 cm wide, and 11 cm high), each one equipped with gravel substrate and a shelter (Fig. 1b). Before a novel environment test (NE) was initiated, two operators collected six experimental animals and placed them individually within the test tanks inside a PVC cylinder (Fig. 1b). After one minute, PVC cylinders were simultaneously removed, and the juvenile lobster were free to explore the novel environment for a total of 5 min.

Soon after the test ended, three juvenile seabreams (10 ± 1 cm in length) were released into each of the two squared, central tanks, and the predator avoidance test (PA) was initiated. The behaviour of each individual lobster was recorded for further 5 min while in the presence of the predators (Fig. 1b). Notably, transparent and perforated walls divided the predator arenas from the lobsters' test tank, so that lobsters were able to both visually and chemically perceive the presence of their predators, which is known to increase the perceived predation risk in prey animals than when they are exposed to simpler, unimodal cues (Ward & Mehner, 2010). At the end of the PA test, the juvenile lobsters were moved back into their housing containers and the test tanks were emptied and filled with clean water before the next trial commenced.

We chose activity (time spent moving, in sec) and refuge use (time spent inside the shelter, in sec) as the reference traits (Polverino et al., 2018 and 2021), since individual-level variation in these behaviours is a target of selection in non-sessile animals (Réale et al., 2007) and have ecological and evolutionary consequences (Wolf & Weissing, 2012).

Resident-intruder test

The resident-intruder test (RI) was performed on 96 of the experimental individuals (randomly selected and balanced across treatments) once they reached the seventh benthic stage, that is, approximately 26 weeks since hatching (Fig. 1b). Individuals were assayed in their individual rearing containers, to test for their territoriality (Karnofsky & Price, 1989). To do so, 50 juvenile lobsters were used as the intruders, and were comparable in age and size to the focal individuals.

Before a RI trial started, a selected intruder was placed inside a perforated transparent cylinder located in the middle of the compartment. After 20 sec the transparent cylinder was gently lifted, and the intruder was free to physically interact with the resident lobster for up to two consecutive min (Fig. 1b). To minimise distress in the animals during the trials, a test was terminated soon after the first attack was observed. Trials were videorecorded with a GoPro Full HD camera (48 frame per sec) located 50 cm above the grid. Subsequently, the latency of the resident lobster to attack the intruder was recorded. Each intruder was changed after each trial, and a new intruder was randomly chosen so that focal animals never encountered the same intruder twice. In order to obtain more accurate result and evaluate the consistency of behaviour over time, each individual was assessed twice, with tests four days apart.

Morphometric measurements

Each juvenile was photographed with a Fujifilm xp140 camera and its carapace length (CL, mm) was measured with the dedicated software imageJ (Schneider et al., 2012), blind to the treatment, before each round of behavioural tests. Therefore, we collected three morphometric datapoints per individual. Additionally, we determined the period of time between two consecutive moults (i.e. intermoult period, IP, days) per each individual.

Data analysis

A final sample size of 250 juvenile lobsters (control: n=63; substrate: n=61; shelter: n=62; substrate + shelter: n=64) out of 256 individuals completed all behavioural and morphometric assays—six juveniles were lost due to early mortality—for a total of 1.536 behavioural observations. Instead, 96 individuals performed the RI test, for a total of 192 behavioural observations. Overall, our sample size relied on > 130 h of video recordings.

Data analysis was performed with *RStudio* v. 4.2.1 (*R* Core Team, 2016), using the packages *lme4, lmerTest,* and *emmeans* (Bates, 2014; Kuznetsova et al., 2017; Lenth et al., 2023). We assumed Gaussian error distribution, which was confirmed for all response variables after visual inspection of model residuals. The significance level was set at $\alpha < 0.05$.

We were interested to test whether and how the presence of environmental stimuli during development altered mean behaviours and life-history traits of the animals, as well as their group-level plasticity. To do so, we fitted generalized linear mixed-effects models with activity (i.e. time spent moving, in s), refuge use (i.e. time spent inside the shelter, in s), body size (i.e. carapace length, in mm), and intermoult period (i.e. time between two consecutive moults, in days) as the dependent variables, respectively. In the fixed-effect structure of the models for activity and refuge use we included rearing treatment (presence/absence of a shelter and/or substrate; four levels), predator treatment (predator cues and no predators), test (novel environment and predator avoidance), grid (four levels), mother ID (four levels), stage (three levels), test duration (in sec), and the interaction between rearing treatment, predator treatment, and test. In the aggressiveness model we included rearing treatment, predator treatment, their interaction, grid, and mother ID as the fixed effects. The models for carapace length (CL) and intermoult period (IP) included, instead, rearing treatment, predator treatment, stage, their (three way) interaction, grid, and mother ID as the fixed effects.

We ran pairwise comparisons with the conservative Bonferroni method for significant predictors, while accounting for the variation explained by other predictors.

We included individual identities (random intercepts) in the random structure of all models to account for repeated measures. The aggressiveness model also included intruder identities as random intercepts. Since individuals might differ in the way that they adjusted their activity and refuge use across tests, developmental stages, and treatments or as function of their mother ID, we also tested for the presence of heterogeneous variance between tests, developmental stages, rearing treatments, predator treatments, and mother IDs (that is, random slopes/regression). We did this by running five separate models for activity and refuge use in which we included random intercepts and slopes one-by-one. Then, we used both likelihood ratio tests (LRTs) and Akaike information criteria (ΔAIC) to compare models with different random intercepts and slopes and chose models with the best likelihood ratio, and lower AIC. With the same approach, we also tested whether random intercepts explained a significant portion of the variation observed, that is, whether individual variation in activity and refuge use was highly structured (Dingemanse & Dochtermann, 2013).

Results

The rearing conditions to which individuals were exposed during their early development had a strong effect on the activity levels and aggressiveness of the animals, with marginal, albeit non-significant, effects also on the growth rate of the individuals (i.e. intermoult period; Table 1). In particular, the presence of both substrate and a shelter in the housing enclosure resulted in individuals being more active than their siblings raised under both control conditions and in the presence of substrate only (Fig. 2a). Instead, juveniles raised with both substrate and shelter, or with substrate only, were less aggressive toward intruders (i.e. longer latencies to attack) than conspecifics raised under control conditions (Fig. 2c). On the contrary, no significant effects of rearing conditions were observed for refuge use and carapace length (Fig. 2b).

Chronic exposure to the predator cues had a negative impact on the carapace length of the individuals, with juveniles from the predator treatment being on average smaller than those not exposed to predator cues (Fig. 3a). Overall effects of predator exposure were less evident on the other traits. However, we observed that the interaction between rearing conditions and predator cues explained a large portion of the behavioural variance observed (Figs. 2a, 2b). For instance,

juveniles that had access to shelters during development, or that were raised in the presence of both a shelter and substrate, were more active and aggressive than control individuals (Figs 2a, 2c). On the contrary, effects of the rearing treatment partly disappeared when individuals were simultaneously exposed to predator cues during their development (Figs 2a, 2b).

The behavioural and life-history traits of the individuals varied, on average, across ontogenetic stages (Table 1; Fig. 3b). For instance, activity levels decreased, and refuge use increased over development. We also observed that intermoult period (i.e. growth rate) of the individuals varied between ontogenetic stages depending on the rearing treatment experienced by the individuals (Fig. 3b): the time between two consecutive moults did not differ between rearing treatments early in life, but individuals reared in the presence of substrate and/or a shelter (but not both substrate and shelter together) grew faster than control animals later in life (Fig. 3b). As expected, activity levels and refuge use varied between tests (novel environment, NE, and predator avoidance, PA; Table 1): individuals spent on average less time exploring the experimental arena and hid longer in their refuge when live predators were present (PA) than in the absence of predator cues (NE).

We found evidence that the inclusion of random intercepts (i.e. individual IDs) and test and stage as random slopes increased model fit (Table 2). In other words, individuals differed in behaviour among each other consistently over time (i.e. behavioural individuality), and also differed from each other in the way that they adjusted their activity levels and refuge use across tests and developmental stages (i.e. behavioural plasticity). Instead, no differences were detected between models with rearing treatment, predator treatments, or mother IDs as the random slopes, and we did not retain these terms in our final models (Table 2).

Discussion

Our analysis provides solid evidence that early-life exposure to structural and sensory stimuli in the rearing environment have important effects on both the average and the individual-level behaviours and life-history traits of European lobsters. For instance, the presence of a shelter in the housing structure resulted into higher activity rates of the individuals, while the presence of a gravel substrate reduced their aggressiveness toward intruders. Instead, individuals reared in the presence of either the gravel substrate or the shelter grew faster later in life than control animals, while the exposure to predator cues during development resulted into smaller body sizes of the lobsters.

A main result of this work is that structural enrichments in the rearing enclosures resulted into higher activity levels and lower aggressiveness in the animals. Studies on other aquatic organisms indicate that the presence of environmental enrichments in the rearing enclosures enhances the neural plasticity of these animals by increasing the dopaminergic and serotonergic activity that, in turn, promotes exploratory behaviours, spatial orientation, and learning capacities (Newberry, 1995; Ullah et al., 2017; Arechavala-Lopez et al., 2020). Similarly, structural enrichment was found to reduce aggressiveness in captive-bred fishes (*Tilapia rendalli*) by Torrezzani and collaborators (2013). In fact, it is reasonable to believe that habitat complexity provides opportunity for individuals to establish territories and safe refugia, thereby reducing basal stress and attenuating their aggressive behaviours. In line with this interpretation, our lobsters raised in the presence of substrate and/or shelter also grew faster (i.e. had lower intermoult periods) later in life, than control groups. Although, to the best of our knowledge, there are no studies that have explored the effects of environmental enrichments on the intermoult period of lobsters, existing research on aquatic organisms indicates that enriched environments reduce stress levels and favour lower resting metabolic rates of the animals, which, in turn, reduce the energy expenditure of the body machinery and favours growth (Howell & Canario, 1987; Millidine et al., 2006; Marcon et al., 2018; Zhang et al., 2021). It is therefore conceivable that environmental enrichment has contributed, over the course of development, to a decrease in stress levels and, consequently, an increase in growth rates, manifested through shorter intermoult periods of the lobsters. Notably, we observed a consistent pattern of longer refuge use and lower activity levels with advancing age, suggesting a shift toward a properly benthic behaviour over development that is well known for these animals (Botero & Atema, 1982; Cobb et al., 1989; Castro & Cobb, 2005; Latini et al., 2023).

Interestingly, our work points out that the exposure to predatory cues during development can result into a smaller body size of the individuals (i.e., carapace length). According with classic life-history theory, in which different predatory regimes result in population having different life-history strategies (Reznick & Endler, 1982), a reduction in body size can be interpreted as a trade-off between securing resources and reducing chances of being predated (Houston et al., 1993; Krause et al., 1998; McNamara et al., 2005). In fact, prior evidence indicates that prey tend to reduce activity levels and increase refuge use in response to the presence of a predator (Lima & Dill, 1990; Sih & McCarthy, 2002). In support of this interpretation, we observed that individuals spent on average more time hiding in the refuge when tested in the presence of their predators (PA test) than when predators were absent (NE test). But effects of the predator treatment were not only

related to changes in the life-history traits of the animals, and shaped even the behavioural profiles of our individuals. Specifically, the exposure to predator cues during development reduced the beneficial effects of gravel substrate and shelter on the lobsters' behaviour: individuals were more active and risk prone (i.e. lower refuge use) when raised with substrate and shelter than conspecifics reared in absence of such structural enrichments, but effects tapered away in animals also exposed to predatory cues during development. These findings suggest that when environmental stimuli are combined, their interaction may lead to effects that do not necessarily correspond to those observed when only one enrichment is present (Näslund, 2021).

Ultimately, our results revealed that individuals consistently displayed distinct behaviour over time (i.e. behavioural individuality), alongside variation in the modulation of their activity levels and refuge use (i.e. behavioural plasticity) depending on the presence/absence of predators in their surrounding environment and developmental age. These findings indicate that the early-life environment plays a crucial role in shaping phenotypic variation within animal groups, and impact their ability to respond to rapid environmental changes (De Gasperin et al., 2019; Zocher et al., 2020). Since phenotypic variation among individuals of a population are likely to increase over development (Polverino et al., 2016), it becomes imperative to carefully plan rearing conditions during juvenile stages of lobsters to foster diversity in the phenotypic traits at adulthood and enhance the likelihood of post-release success. While it remains unknown whether these experimentally-induced phenotypic variations will persist over time and after animals are released in the wild, the literature suggests that individual-level measurements performed in the lab well reflect the natural variation observed among the individuals once released into the wild (Laskowski et al., 2016).

A key goal in conservation aquaculture is producing animals that can effectively survive and reproduce in the wild. We propose that introducing ecological-relevant stimuli in the rearing environment can benefit conservation programs by promoting natural diversity in animal phenotypes, which is essential for thriving under challenging environmental conditions.

Fig. 1 - Representation of the experimental procedure. a) Ontogenetic treatments. Juveniles raised in individual compartments were exposed to different rearing treatments that differed for the presence/absence of environmental stimuli: shelter, substrate, and predator cues (1h per day). Juveniles were subjected to these conditions from the beginning of their benthic life for approximately 24 consecutive weeks. **b)** Acute treatments and behavioural tests: novel environment and predator avoidance. The setup consisted of two squared, central areas to house the predator during the predator avoidance test, and six visually isolated rectangular compartments (lobster's tanks) equipped with substrate and a shelter. The resident-intruder test was performed at the seventh benthic stage on a subsample of the animals.

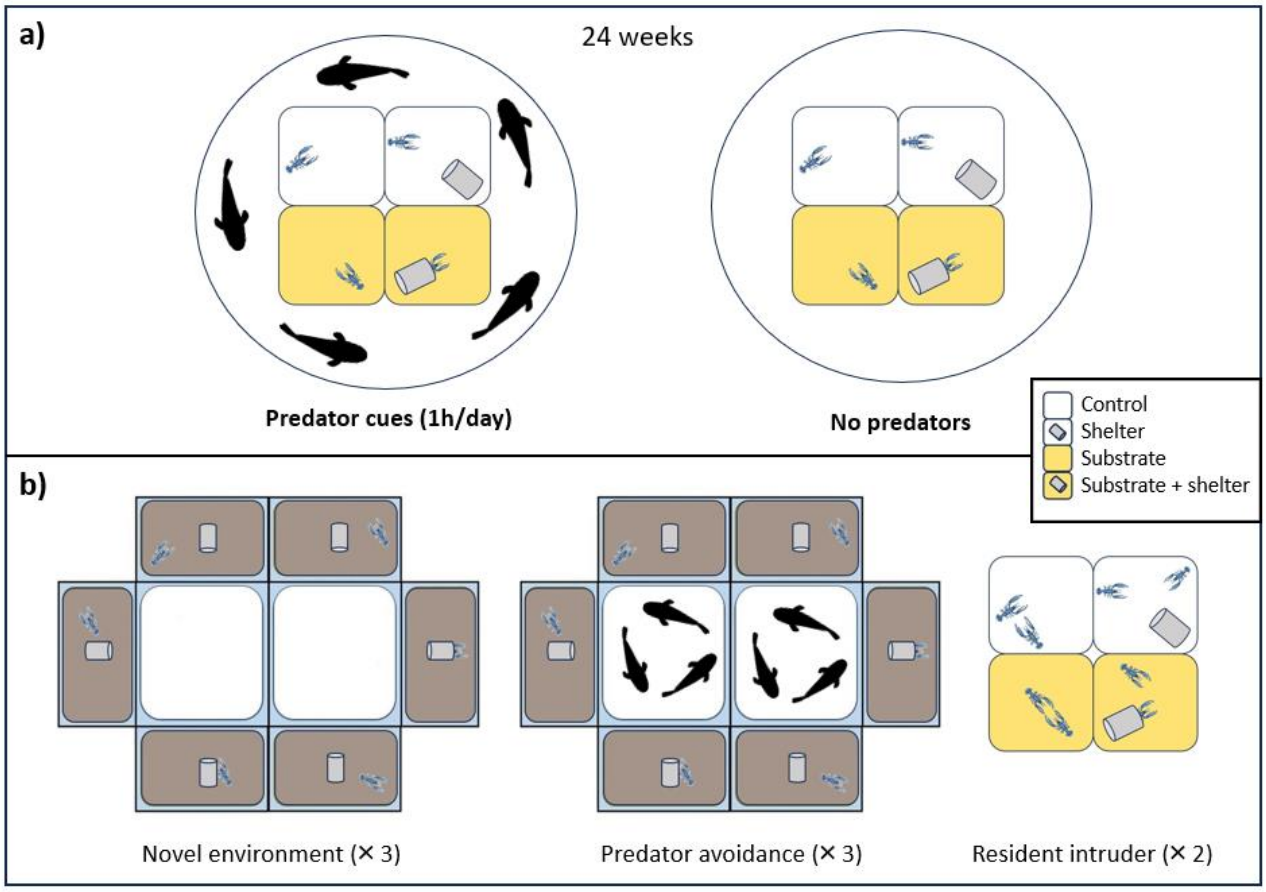


Fig. 2 - Effects of the different rearing conditions on the expressed behaviours. In (a) and (b) are respectively represented the treatment effects on activity and refuge use, and in (c) the effect on aggressiveness expressed as latency to attack.

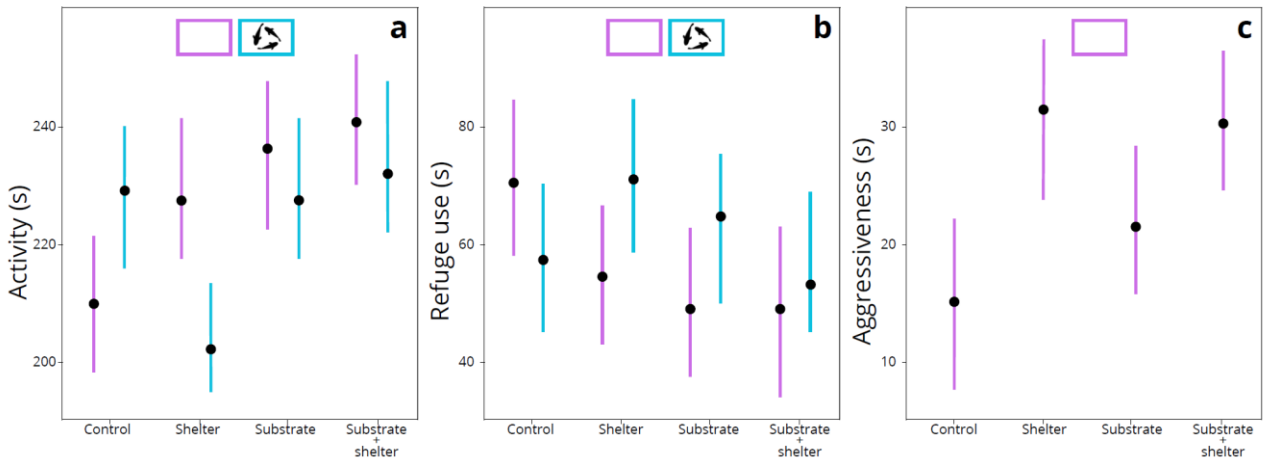
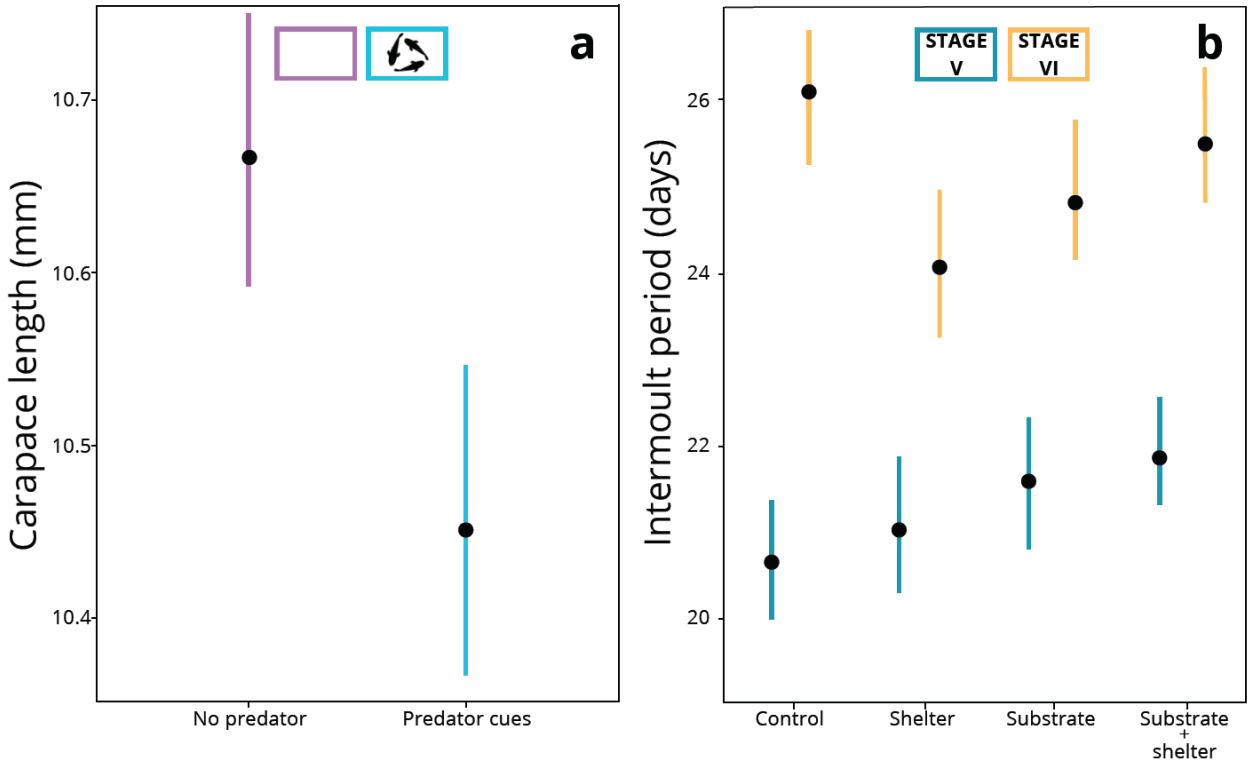


Fig. 3 - Effects of the different rearing conditions on life-history traits. In (a) is reported the mean effect of predator cues on carapace length, and in (b) the effect of rearing treatment on the intermoult period of the individuals.



Tab. 1 - Results from the fixed-factor structure of the generalized linear-mixed models. Rearing treatment (four levels), predator treatment (two levels), test (two levels), grid (four levels), mother ID (four levels), stage (three levels), test duration (in sec), and the interaction between rearing treatment, predator treatment, and test are included as fixed effects in the models for activity and refuge use. In the aggressiveness model rearing treatment, predator treatment, their interaction, grid, and mother ID were the fixed effects. For the life-history traits CL and PI, rearing treatment, predator treatment, stage, their (three way) interaction, grid, mother ID, and stage are included as the fixed effects. Significance was $\alpha < 0.05$ and significant results are in bold.

		Mean Sq.	*df*	*F*	*P*
Activity	Rearing	24551	3, 397	5.464	**<0.001**
	Predator	1302	1, 428	0.290	0.590
	Test	1645111	1, 483	366.714	**<0.001**
	Grid	8847	2, 453	1.972	0.140
	Mother ID	2793	3, 454	0.623	0.600
	Stage	346274	2, 537	77.188	**<0.001**
	Duration	26743	1, 1281	5.961	**0.015**
	Rearing × predator	15704	3, 398	3.500	**0.016**
	Rearing × test	24764	3, 805	5.520	**<0.001**
	Predator × test	581	1, 804	0.130	0.719
	Rearing × predator × test	6346	3, 806	1.414	0.237
Refuge use	Rearing	6955	3, 481	1.666	0.173
	Predator	568	1, 510	0.136	0.712
	Test	660251	1, 472	158.204	**<0.001**
	Grid	2127	2, 531	0.510	0.601
	Mother ID	2317	3, 532	0.555	0.645
	Stage	24366	2, 518	72.406	**<0.001**
	Duration	26743	1, 1280	5.838	**0.016**
	Rearing × predator	10540	3, 481	2.526	0.056
	Rearing × test	10066	3, 890	2.412	0.065
	Predator × test	132	1, 889	0.032	0.860
	Rearing × predator × test	3406	3, 891	0.816	0.485
Aggressiveness	Rearing	2267.42	3, 84	5.485	**<0.001**

	Predator	4.64	1, 85	0.011	0.916
	Grid	637.45	2, 56	1.542	0.222
	Mother ID	378.46	3, 80	0.915	0.437
	Rearing × predator	338.06	3, 81	0.818	0.488
Carapace length	Rearing	0.360	3, 244	1.128	0.334
	Predator	4.100	1, 244	12.901	**<0.001**
	Grid	0.590	2, 244	1.854	0.156
	Mother ID	10.220	3, 244	32.187	**<0.001**
	Stage	839.420	2, 491	2644.197	**<0.001**
	Rearing × predator	0.330	3, 244	1.039	0.376
	Rearing × stage	0.550	6, 491	1.744	0.110
	Predator × stage	0.170	2, 491	0.537	0.585
	Rearing × predator × stage	0.150	6, 491	0.485	0.819
Intermoult period	Rearing	13.340	3, 237	2.436	0.065
	Predator	0.090	1, 237	0.017	0.896
	Grid	7.650	2, 237	1.396	0.249
	Mother ID	4.350	3, 237	0.793	0.499
	Stage	1933.660	1, 242	353.033	**<0.001**
	Rearing × predator	13.130	3, 237	2.397	0.069
	Rearing × stage	37.130	3, 242	6.779	**<0.001**
	Predator × stage	0.99	1, 242	0.181	0.670
	Rearing × predator × stage	10.27	3, 242	1.875	0.134

Tab. 2 - Comparison of LMMs with different random (co)variance structures for activity (time spent in activity) and refuge use (time spent inside the shelter). We compared two models with both random intercepts (ID) and slopes (test, predator treatment, rearing treatment, mother ID, or stage) across individuals against a reduced model with only random intercepts. We also compared the model with random intercepts with a model in which random intercepts were excluded. Significant improvements in the model fit are tested with both Akaike information criteria (ΔAIC) and likelihood-ratio tests (*P*). Rearing treatment (control, substrate, shelter, substrate + shelter), predator treatment (predator cues, no predators), grid (four levels), mother ID (four levels), stage (three levels), test duration (in sec) are included as fixed effects in models considered for activity and refuge use. Significance was set at $\alpha < 0.05$ and significant results are in bold; the model with random intercepts and test and stage as the random slope was best supported as the most parsimonious model for activity, while random intercepts and stage as the random slopes explained a significant portion of the variance in refuge use. The random structure is written in the syntax of the *lme4 R* package.

		X2	ΔAIC	*P*
Activity	(test|ID)	29.506	25	**<0.001**
	(predator treatment|ID)	5.891	2	0.053
	(rearing treatment |ID)	2.222	16	0.987
	(mother ID|ID)	3.742	14	0.927
	(stage|ID)	108.000	104	**<0.001**
Refuge use	(test|ID)	29.506	25	**<0.001**
	(predator treatment|ID)	5.979	2	0.051
	(rearing treatment|ID)	6.488	12	0.690
	(mother ID|ID)	13.169	5	0.155
	(stage|ID)	175.110	171	**<0.001**

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5. Conclusions

This research aimed to examine how the captive early life environment influences the expression of phenotypic traits and their plasticity. Specifically, by investigating the phenotypic plastic responses of juvenile European lobsters (*Homarus gammarus*) subjected to diverse hatchery-rearing conditions, this research project seeks to promote the development of wild-like phenotypes and enhance their overall variability, with potential benefits for conservation programs.

In chapters 3 and 4, I explored the ability of hatchery reared juvenile lobsters to express different phenotypes in response to variations in early developmental rearing conditions. It was found (chapter 3) that the size of the individual holding spaces triggered a plastic response, influencing both growth and behavioural performances. Specifically, confining lobsters to restricted holding spaces limits and slows down their overall growth while compromising the development of ecologically-relevant behaviours that are essentials for these animals to survive in a complex and risky environment (Botero & Atema, 1982; Castro & Cobb, 2005; Mehrtens et al., 2005). Moreover, I noted that juveniles demonstrated specific behavioural responses at different developmental stages, which can be attributed to variations in their rearing conditions. Similarly, differences on growth performances between juveniles belonging to the two treatments were not equal for each developmental stage but tended to become more pronounced with increasing age. These results emphasize that individual holding space not only influences overall phenotypic expression but also induces diverse phenotypic responses during particular developmental stages, underscoring the importance of identifying the most suitable ontogenetic period for the implementation of specific rearing procedures (Strand et al., 2010; Salvanes et al., 2013).

Then (chapter 4), I examined how early-life exposure to varying levels of environmental complexity (presence/absence of substrate and/or shelter) and predator stimuli influences the development of plasticity and individuality in ecologically relevant behaviours and life-history traits of hatchery-reared juvenile lobsters. The obtained results highlight that exposure to a single environmental stimulus or the interaction between multiple stimuli during early development differently influences both the average expression of behavioural and life-history traits and their individual variation. These findings suggest that when environmental stimuli are combined, their interaction may lead to effects that do not necessarily correspond to those observed when only one enrichment is present (Naslund, 2021), and further indicate that plasticity in response to one environmental variable can interact with plasticity in response to another, producing a combined

effect on the expressed traits (Mitchell & Biro, 2017). Additionally, they provide novel experimental evidence emphasizing the crucial role of early life experiences in shaping phenotypic differences among individuals and in altering their ability to respond to rapid environmental changes.

Overall, the results obtained from these two studies suggest that relatively simple modifications of rearing environments during the early-developmental stages can significantly alter the ability of individuals to rapidly express different phenotypes in response to specific stimuli. Here, the focus has been on holding space, physical enrichment, and presence/absence of predator stimuli, three critical features of the captive environment that can mediate phenotypic effects (Reading et al., 2013; Toni et al., 2019; Kopack et al., 2023). Thus, it can be concluded that if environmental modifications are adequately adapted to species and life stage-specific conditions, they can promote phenotypic plasticity and the expression of wild-like phenotypes likely ameliorating the post-release performance of hatchery-reared juveniles. In addition, adequate conditions of the rearing environment are fundamental for improving animal welfare, which is not only an ethical imperative but also provides multiple benefits: from the quality of the products to the success of the research and stocking activities (Conte, 2004; Segner et al., 2019; Carere & Mather, 2019).

As regard future directions, it is crucial to delve deeper into epigenetics mechanisms, which examines how gene expression is altered without modifying the DNA sequence (Berger et al., 2009). This involves understanding the roles of DNA methylation, histone modifications, and regulation by non-coding RNA in shaping cellular responses to environmental changes (Al Aboud et al., 2023). Unravelling these complexities is essential for elucidating the extent and mechanisms through which gene expression can adapt to different conditions, ultimately leading to the acquisition of plastic traits (Duncan et al., 2014). Furthermore, while controlled hatchery and laboratory experiments are useful, coupled field studies are invaluable to assess the effectiveness of pre-release conditioning, to identify interacting factors and to reveal plastic traits that are ecologically important (Daly et al., 2021). Additionally, further research aimed at improving rearing methodologies for conservation purposes should prioritize investigating the impact of captive environments on individual-level variation rather than solely concentrating on the study of average effects. Ignoring both inter and intra-individual phenotypic variations implies overlooking factors that affect population performance, community interactions and ecosystem function (Hughes et al., 2008; Bolnick et al., 2011), with important implications for management and conservation outcomes (Huges et al., 2019). Consequently, understanding how diverse rearing conditions may influence individual trait

variation represent a substantial challenge for the future management of conservation aquaculture programs, and is also a noteworthy consideration for animal welfare (Webster, 1995; Zhang et al., 2019).

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