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"Transgenerational effects of temperature on egg and larval development in *Cyprinodon variegatus*"

Tesi di laurea in ADATTAMENTI DEGLI ANIMALI ALL'AMBIENTE MARINO

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1. INTRODUCTION

1.1 CLIMATE CHANGE

The Earth's climate has changed before, but this time is different. People are causing these changes, which are bigger and happening faster than any climate changes modern society has ever seen before. Heat-trapping greenhouse gases exist naturally in the atmosphere and help keep the Earth warm enough for flora and fauna to live. Without greenhouse gases trapping heat in the atmosphere, our planet would be a very cold place. However, temperatures are increasing because people are adding extra greenhouse gases (carbon dioxide, methane, nitrous oxide, flourinated gases and other gases) to the atmosphere, mainly by burning fossil fuels.

Greenhouse gases come from everyday activities, heating our homes, driving around town, and using electricity. Moreover, these greenhouse gases don't stay in one place after they're added to the atmosphere, so even though some countries produce more greenhouse gases than others, emissions from every country contribute to the problem and climate change requires global action. There's more carbon dioxide in the atmosphere now than at any other time in at least 650 000 years, and the amount of carbon dioxide and other greenhouse gases is continuing to increase.

The warmer temperatures are causing other changes around the world, such as ocean warming, rising sea levels, ocean acidification, expansion of oxygen minimum zones, changing rain patterns, more heat waves, droughts and wildfires, floods, loss of wildlife habitats, changes in animal migration and life cycles, changes in primary productivity and ocean circulation patterns, variation in resting metabolic rates (Donelson et al. 2011), altered foodwebs, extinction, melting glaciers, thawing permafrost, stronger and more frequent storms and hurricanes. These changes are happening

because Earth's air, water, and land are all related to one another and all linked to the climate (Fig. 1).

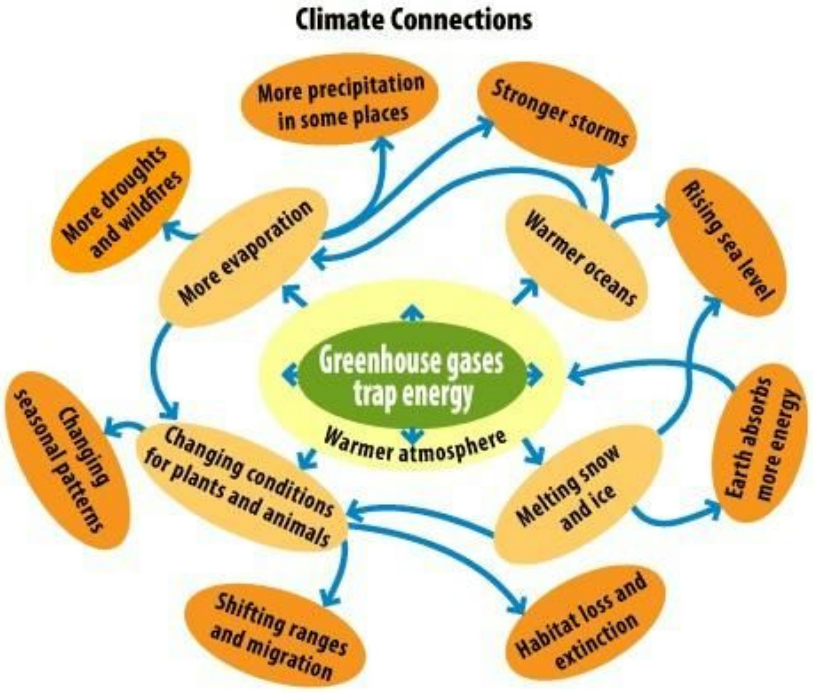

Fig. 1. Simplified climate connections diagram that shows how global warming can lead to a variety of other changes (from www.epa.gov).

We expect serious consequences of Climate change for several ecosystems, resulting in disruptions to ecosystem processes, an overall loss of community diversity, and a reduction in the ecological goods and ecosystem services provided to human societies (Thomas et al. 2004; Lovejoy & Hannah, 2005; Bozinovic et al. 2011).

Climate change will also influence ocean circulation and upwelling. We expect increased thermal stratification of the ocean surface layer, which will reduce the input of nutrients from cooler waters below the thermocline (Bindoff et al. 2007; Poloczanska et al. 2007). Ocean warming, changes to wind fields and increased thermal stability of the shallow surface zone may tend to increase the strength of ocean and surface currents (Steinberg, 2007), which would in turn impact dispersal

patterns and survival of larval fishes (Sponaugle and Pinkard 2004).

Climate change is also likely to increase the risk of fisheries collapses. It will most likely lead to large-scale redistribution of global catch potential, with a drop of up to 40% in the tropics and an average of 30-70% increase in high-latitude regions (Cheung et al. 2010). Climate change affects primary productivity, on which most marine organisms depend as an energy source. Global ocean phytoplankton biomass may have declined substantially over the past 50 years (Boyce et al. 2010). Moreover, the phenology and size structure of planktonic communities is likely to change, leading to mismatches in the timing of ecological interactions, potentially affecting the survival of recruits to fish populations (Ji et al. 2010). Changes in primary productivity and planktonic community structure affect the amount of energy transferred to higher trophic levels and, eventually, the productivity of trophic groups that contribute to fisheries catches (Cheung et al. 2008). Due to the predicted increase in recruitment variability, determining optimal harvest strategies will be more difficult and fisheries are likely to become more susceptible to overfishing during episodes of low recruitment (Munday, 2008). Although marine capture fisheries contribute largely to global animal protein supply, there's a lack of global projections of climate change impacts on marine fisheries (Cheung et al. 2010). Climate change is already and will continue to impact the quantity and quality of marine fish catch and its distribution, thus affecting the economics of fishing, therefore it is critical to adapt fisheries to climate change (Sumaila et al. 2011).

The ecological impacts of climate change are evident, from polar terrestrial to tropical marine environments, and organisms in a wide range of ecosystems and organizational hierarchies are responding on many levels (Walther et al. 2002).

1.1.1 Global warming

While climate change refers to the broader set of changes including changes in weather patterns, the oceans, ice and snow, and ecosystems, etc., “Global warming” refers specifically to the increase in the average temperature near the Earth's surface.

Over the past 100 years, global warming has consisted of a temperature increase of 0.7°C, with two main periods of warming, the first between 1910 and 1945 and the second from 1976 onwards. The warming rate during the second period has doubled that of the first and has been the greatest in the last 1000 years. Less than 1°C may not seem like much, but small changes in Earth’s temperature can have severe effects, some of which are already happening (Walther et al. 2002).

The concentration of CO_2 in the atmosphere has increased from approximately 280 p.p.m. at the start of the industrial revolution to over 390 p.p.m. now. This is the result of fossil fuel burning, cement production and land use changes (Peters et al., 2012).

Global warming impacts both the mean temperature of local environments and the magnitude of seasonal and diel temperature variation. Moreover, thermal variance could have as much, or even more, of an impact on fitness as the mean temperature (Bozinovic et al. 2011).

Temperature deeply affects biological functions from molecular to ecosystem levels (Hochachka & Somero, 2002). Therefore, it is thought to be one of the critical abiotic factors influencing the distribution and abundance of organisms (Schulte et al. 2011).

Water has a higher heat capacity than air, therefore, for the same heat input, ocean temperature increases more slowly than air temperature. Depending on future CO_2 emission scenarios, global average surface temperature is projected to increase 1.1–6.4°C by 2100, with best estimates placing the range between 1.8 and 4.0°C (Munday et al. 2008). Although global warming is

still at an early stage, ecological responses to recent climate change are already clearly visible (Walther et al. 2002).

On a proximate level, global warming could alter the shifting of patterns of activity during climate change and, on an ultimate level, warming could influence selective pressures that shape physiology and behavior in future climates (Sears et al. 2011).

1.1.2 Effects on marine organisms

Understanding and predicting how populations will react to changes in the environment is a long-standing goal in evolutionary ecology.

The geographic range boundaries of marine ectotherms are closely matched to their potential latitudinal ranges, on the basis of thermal tolerance and extreme temperatures across latitudes, and the close coupling between thermal tolerance and environmental temperature suggests that marine species will be sensitive to temperature variation at both their equatorward and poleward range boundaries (Sunday et al. 2012). Therefore, climatic regimes influence species' distributions, often through species-specific physiological thresholds of temperature and precipitation tolerance (Hoffman & Parsons, 1997). We expect species to track the shifting climate and consequently shift their distributions according to the changing climate in a direction that is generally towards higher latitude, poleward, and deeper water, with rates of range shift of 30-130 km per decade (Cheung et al. 2010), to the extent that dispersal and resource availability allow. Studying fish species from the North Sea, Perry et al. (Perry et al. 2005) found that 6 out of 12 species shifted their southern boundary northward. The largest boundary shift was exhibited by blue whiting, *Micromesistius poutassou* (816 km). However, in some cases, such as reef-building corals, range shifts in response to increasing temperature may not be possible if latitudinal distributions are limited by other factors, too

(Hoegh-Guldberg, 1999).

Changes in distributions of plants and animals, in population abundances, adjustments to the timing of seasonal activities, the propagation of invasive species (Stachowicz et al. 2002) and increased prevalence of disease are all linked to climate change (Harvell et al. 2002; Parmesan & Yohe 2003; Root et al. 2003; Parmesan 2006). As the climate changes rapidly over the next 50–100 years, we expect these types of impacts to become more pervasive (Munday et al. 2008).

Critical factors in determining persistence at locations during climate change are Resting metabolic rate (RMR), generally measured as resting oxygen consumption, and maximal oxygen uptake. Specifically, at increased temperatures, the maximum capacity for oxygen uptake can no longer keep pace with the rise in resting metabolism, negatively impacting processes like reproduction or feeding (Portner & Knust, 2007).

Local changes in life history characters are expected to be quite common (Gienapp et al. 2008), including growth rate and lifespan, major determinants of the resilience of a population. Consequently, there is a clear need for robust predictions of the effects of climate change on critical life history traits in local populations.

Over the last 60 years, thermal biology has shifted from a more physiological science to a more integrated science of physiology, ecology, behavior, and evolution. Temperature, especially for ectothermal organisms, has a dominant role in shaping metabolism, lifespan, and population dynamics, therefore it is necessary to understand the responses to changing temperatures. The fact that temperature is one of the strongest variables that affect biological processes and that laws of thermodynamics define the direction and rate of organisms' biochemical processes are fundamental assumptions of thermal biology (Haynie, 2001; Brown et al. 2004). Given these assumptions, the

environment exerts strong selective pressures on all organisms and knowledge of thermal biology is the key to explaining many physiological, ecological, and evolutionary patterns (Angilletta et al. 2006).

Fishes are ectotherms and temperature changes of just a few degrees Celsius can influence their physiological condition, developmental rate, growth rate, swimming ability, reproductive performance and behaviour (Wood & McDonald, 1997). Fishes are very sensitive to temperature during the early stages of their life history. Increased temperature tends to increase larval growth rate, decrease the age at metamorphosis and increase swimming ability (Hunt von herbing, 2002). Provided that the temperatures do not exceed optimum levels for growth and development, and that the larvae can consume a sufficient amount of extra food to support the higher energetic demand of developing, small increases in water temperature will generally speed up the growth and developmental rate of larval fishes and decrease their larval duration. However, increased temperature may also increase mortality rates, indicating that even a relatively small temperature increase could effect the number of larvae reaching settlement negatively (Munday et al. 2008).

Many species use temperature to start the breeding season, but others may use photoperiod or a combination of temperature and photoperiod (Pankhurst & Porter, 2003). These species would be the most impacted by increased temperature because they are less likely to be able to adjust the timing of reproduction to suit the new thermal environment. Reproductive success could be seriously compromised if higher water temperatures altered egg production (Van Der Kraak and Pankhurst 1997), increased embryonic mortality (Gagliano et al. 2007), or lead to a mismatch between the timing of breeding, set by photoperiod, and the optimal conditions in the plankton for survival and dispersal of larvae, set by temperature (Edwards and Richardson 2004).

Range shifts correlated with higher oceanic temperatures have been observed in temperate marine fishes (Perry et al. 2005). As sea temperatures increase, many small-range species could

experience dangerous range contractions. Whether a species will shift its range, how the geographic ranges will change and the consequences on the persistance of species depends on existing latitudinal distributions, temperature tolerances in relation to the current-day range (Portner & Knust, 2007), interactions with other species at the range boundary, the potential for acclimation and local adaptation, dispersal capacity and the availability of suitable habitat outside the existing range (Munday et al. 2008).

We predict tropical ecosystems to be particularly sensitive to global warming because many tropical species, as a result of having evolved in a relatively stable thermal environment, have a more narrow thermal tolerance range (Deutsch et al., 2008). Consequently, even relatively small increases in temperature could exceed the thermal optimum for some tropical species, leading to declines in individual performance that could affect population sustainability, community structure and ecosystem function (Munday et al. 2012).

In water-breathing animals such as fish, a mismatch between increasing oxygen demand at higher temperatures and the capacity of the circulatory and ventilatory systems to supply oxygen to tissues is thought to be associated with thermal tolerance (Fry, 1947; Pörtner & Knust, 2007; Pörtner & Farrell, 2008). According to this hypothesis, the scope for aerobic performance will decline above the optimal temperature range, with consequences for activity levels, growth rate, reproduction and, ultimately, population survival (Farrell, 2009).

Seasonal change in temperature has a profound effect on reproduction in fish. Elevated temperatures truncate spring spawning, and delay autumn spawning. Temperature increases will affect reproduction, but the nature of these effects will depend on the period and amplitude of the increase and range from phase-shifting of spawning to complete inhibition of reproduction. This latter effect will be most marked in species that are constrained in their capacity to shift geographic range.

Studies from a range of taxa, habitats and temperature ranges all show inhibitory effects of elevated temperature albeit about different environmental set points (Pankhurst & Munday, 2011).

Over 99% of all organisms are ectothermic, and these organisms have been and will continue to be thermally challenged by global climate change. These thermal changes have altered the growth, phenology, and distribution of many organisms (Atrill & Power, 2002; Walther et al. 2002; Parmesan & Yohe, 2003).

1.2 ADAPTATION TO CHANGING ENVIRONMENTS

Understanding a species' physiological capacities when forecasting its response to climate change and the likely influence that capacities for genetic change across generations and changes in plastic responses will have on a species response are two inseparable areas of research (Chown et al. 2010). Without an evolutionary perspective, it is difficult to predict the future impacts of climate change and organisms' biological responses over time (Munday et al. 2013).

Determining the capacity of organisms to acclimate and adapt to higher temperatures is key to understand how populations and communities will respond to global warming (Donelson et al. 2011).

Numerous examples have shown that evolution can be remarkably rapid (Shaw & Etterson, 2012). It can no longer be argued that evolution only occurs over timescales that are dramatically different to the present pace of environmental change. Moreover, phenotypic plasticity is increasingly being found to contribute strongly to persistence in the face of climate change (Charmantier et al. 2008; Anderson et al. 2012; Barrett & Hendry 2012), and may help buffer populations against the immediate impacts of climate change and provide time for genetic adaptation to catch up.

Phenotypic plasticity is a crucial phenomenon that may allow organisms to persist in the face

of environmental change and give populations the time to adapt to climate change (Chevin et al. 2010). Phenotypic plasticity is becoming more apparent as species are forced to cope with rapid changes in the environment and is likely to be an important component of adaptive responses for most species. A thorough understanding of phenotypic plasticity is clearly important to better predict shifts in species distributions (Helmuth et al. 2005) or invasions (Chown et al. 2007).

Munday et al. (Munday et al. 2013) suggest that both phenotypic plasticity and genetic adaptation will play key roles in modifying the impacts of climate change on marine organisms and that they could interact in important ways.

Adaptive responses to thermal heterogeneity involve all levels of biological organization from the expression of genes to the behavior of the organism, but these responses occur on different temporal scales. The interactions among levels of organization that link these responses are thought of as a mechanistic cascade, which flows from the biochemical to the organismal levels, and the challenge for biologists is to define the mechanistic links between thermal responses at different levels and to identify their impact on fitness, to understand the evolution of the mechanistic cascade (Angilletta et al. 2006).

The effects of temperature on biological rate processes and on performance traits within the tolerance zone are described by Thermal Performance Curves (Fig. 2). They tend to have the same general shape: the performance, represented by the y-axis, typically increases as temperature increases, then it reaches a maximum at an intermediate temperature, indicated with T_{opt}, and then rapidly decreases (Huey and Stevenson 1979; Huey and Kingsolver 1989, 1993; Angilletta et al. 2002; Angilletta 2009).

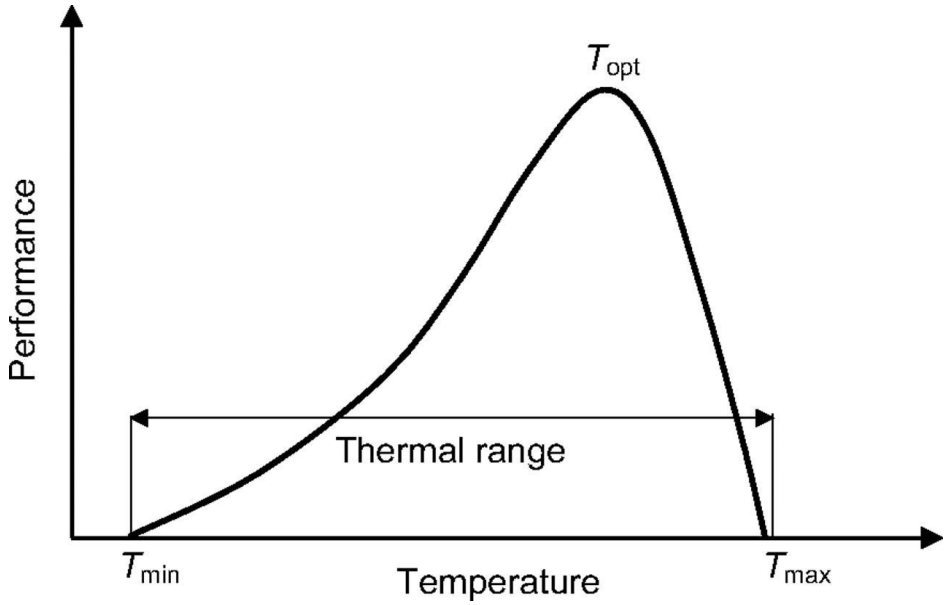

Fig. 2. Hypothetical Thermal Performance Curve (TPC). The temperature at which performance is maximized is termed the T_{opt}. The points at which performance is zero are termed the critical temperatures (Tc, Tcrit, or CTmax and CTmin, depending on the author). TPCs can also be used to define the thermal breadth, which is the range of temperatures at which performance meets or exceeds an arbitrary threshold (often 80% of performance at Topt).

A TPC can be modified by adaptive evolution or phenotypic plasticity. These can alter its height through a vertical shift of the curve, the position of the Topt, through a horizontal shift of the curve, and the width (or breadth) of the curve (Huey and Kingsolver 1989; Angilletta et al. 2003; Izem and Kingsolver 2005; Frazier et al. 2006; Angilletta 2009; Kingsolver 2009), as well as alter the shape of the curve (e.g., linear, exponential, polynomial) in the rising or descending phases. However, this last possibility has not often been investigated.

There has been a recent resurgence in interest in TPCs because of their potential utility in helping to predict the responses of populations or species to climate change (Deutsch et al. 2008;

Angert et al. 2011; Dell et al. 2011; Huey & Kingsolver 2011). TPCs can be incorporated into mechanistically based models of responses of organisms to climate change (Helmuth et al. 2005; Kearney 2006; Kearney and Porter 2009; Angert et al. 2011), which may prove useful for predicting the effects of climate change on species persistence (Kearney 2006; Buckley et al. 2010; Chown et al. 2010).

Thermal acclimation is one way to cope with increased temperature. It involves the altering of behavioral, physiological or morphological characteristics to have a phenotype, complex product of interaction between genotype and environment, that better suits a new environment (Fry, 1967).

When there's a spatial and temporal variation of the environment's temperature, one of four conditions must occur. Either body temperature is regulated by behavior and physiology, or body temperature fluctuates while performance is not impaired, or body temperature fluctuates and performance is initially impaired but then restored by acclimation, or, lastly, body temperature fluctuates and performance is impaired and is not restored by acclimation. Any of these outcomes could be adaptive in certain environments. Each outcome results from a combination of behavioral and physiological strategies, therefore natural selection should produce organisms with suites of traits that are coadapted to the environment. Great efforts have been expended to understand global climate change's ecological consequences. However, the evolutionary consequences remain less clear. Evolutionary responses will determine future interactions among organisms. This interplay between ecological and evolutionary responses will be complex because certain species will evolve more rapidly than others. Knowledge of the rates and magnitudes of potential evolutionary responses will help us to assess the long-term consequences of global climate change. Thus, a game theory of thermal coadaptation might ultimately lead us to understand how to manage biodiversity successfully in the near future (Angilletta et al. 2006).

Some fish species may acclimatize to increased sea temperatures as a result of existing phenotypic plasticity in their populations. Moreover, connectivity between populations should promote some genetic adaptation to increased temperature by gene flow from populations that live in warmer locations. Local adaptation to climate change will be most evident in small, short-lived species, where selection can operate over a large number of generations. Larger species, with long generation times, have little potential for local genetic adaptation to rapid climate change. Also, habitat loss caused by climate change could have a negative effect on reef fishes' potential for adaptation to environmental change, because smaller and more fragmented populations will have less genetic variability for selection to act upon and will be less effective in spreading favourable genotypes (Munday et al. 2008).

Donelson et al. (Donelson et al., 2012) found evidence for developmental and transgenerational acclimation to elevated temperature in coral reef fishes. Despite having little capacity for reversible acclimation as adults, some coral reef fishes have considerable capacity for transgenerational thermal acclimation. The full potential to cope with rising ocean temperature may take several generations to be expressed, therefore this has very important consequences for climate change research. We still have to identify the mechanisms responsible for the great improvement in aerobic performance between generations observed by Donelson et al. (Donelson et al., 2012), but we think they could be associated with variation in epigenetic state (Jablonka & Raz, 2009). Specifically, exposing parents to increased temperature, variations in gene expression and cellular function may prime their offspring to develop more efficient physiological processes for a warmer environment.

Global warming is a current and future issue and we expect ocean temperatures to affect many marine organisms. Adaptation or acclimation though could allow organisms to adjust to higher

temperatures. Even though acclimation of physiological processes usually occurs within a generation, parental effects may facilitate acclimation between generations too (Donelson et al. 2011).

A rapidly emerging field of biological and evolutionary study is non-genetic inheritance (Bonduriansky, 2012), with many examples of the environmental conditions experienced in one generation having important consequences for future generations (Jablonka & and Raz, 2009). These effects could be critically important to the adaptation of species to a rapidly changing climate (Bonduriansky et al., 2012). This topic should be an important area for future experimental investigation in climate change research.

Much of the previous work on phenotypic plasticity focused on what can be termed developmental plasticity, where the environmental conditions experienced during development result in a switch between alternative fixed phenotypes in the adult organism (Kinne 1962). Plasticity may also act at very long time scales, as the environment experienced by a parent has been shown to affect the phenotype of its offspring, even across multiple generations, in a phenomenon that has been termed transgenerational plasticity (TGP).

1.2.1 Epigenetics

Epigenetics is the study of the processes that underlie developmental plasticity and canalization and that bring about persistent developmental effects in both prokaryotes and eukaryotes. At the cellular level, these are the processes involved in cell determination and differentiation. At higher levels of biological organization, epigenetic mechanisms generate the context-dependent, self-sustaining interactions between groups of cells that lead to physiological and morphological persistence. The regulatory mechanisms that establish and maintain variant cellular and organismal states are known as epigenetic control mechanisms, or epigenetic control systems

(Nanney, 1958).

Epigenetic inheritance is a component of epigenetics. It occurs when phenotypic variations that do not stem from variations in DNA base sequences are transmitted to subsequent generations of cells or organisms. Many of the discoveries about epigenetic inheritance between organisms are derived from studies in developmental biology that look at inheritance in cell lineages within an organism. Cell heredity in mitotically dividing cells underlies the persistence of determined states in multicellular organisms. The same cell heredity mechanisms that have been found in cell lineages during development are also observed when epigenetic inheritance occurs between generations of individuals. Epigenetic inheritance in the broad sense is the inheritance of developmental variations that do not stem from differences in the sequence of DNA or from persistent inducing signals in the present environment. As well as cell-to-cell transmission of epigenetic variations in unicellular and multicellular organisms, the definition covers body-to-body (or soma-to-soma) information transference that can take place through social learning, symbolic communication and developmental interactions between mother and offspring. Cellular epigenetic inheritance is a narrower aspect of epigenetic inheritance as discussed in the broad sense. It refers to epigenetic transmission in sexual or asexual cell lineages, and the unit of this transmission is the cell. We define cellular epigenetic inheritance as the transmission from mother cell to daughter cell of variations that are not the result of differences in DNA base sequence and/or the present environment. Transmission can be through chromatin marks, through RNAs, through self-reconstructing three-dimensional structures, and through self-sustaining metabolic loops (Jablonka et al. 1992; Jablonka and Lamb 1995, 2005, 2007a). It occurs following cell division in prokaryotes, mitotic cell division in the soma of eukaryotes, and sometimes following the meiotic divisions in the germline. In Figure 3, we illustrate the difference between the broad and narrow sense of epigenetic inheritance by showing the main routes of

between-generation transmission in a sexually reproducing multicellular organism.

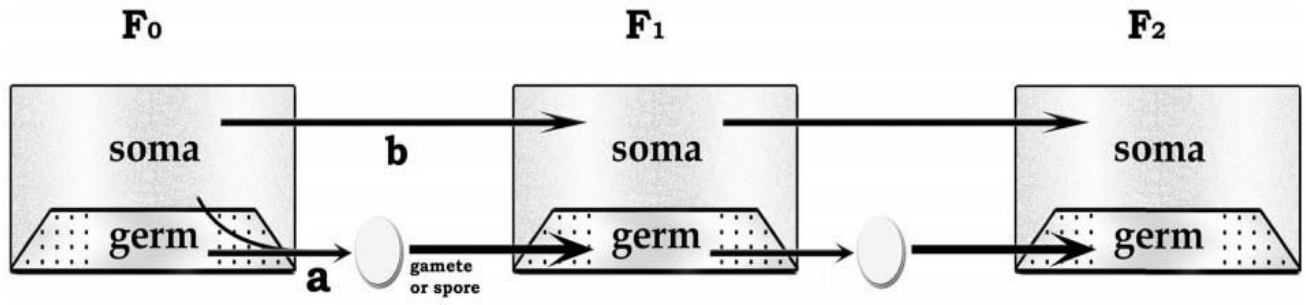

Fig. 3. Routes of Transmission of Epigenetic Variations in a Multicellular, Sexually Reproducing Organism. Route **a** shows the germline-to-germline transmission of induced epigenetic variations (e.g., chromatin marks). A variation can be induced in the germline and can then be transmitted from one generation to the next, or it can first be induced in the soma, then affect the germline, and thereafter be inherited through the germline.

Route **b** shows soma-to-soma transmission (for example, through the transmission of symbionts and parasites, or through the self-perpetuating effects of maternal behavior, social learning, and symbolic communication). A broad view of epigenetic inheritance encompasses both routes a and b, whereas the narrower, cellular view includes only route a—transmission through a single-cell "bottleneck," in this case, a gamete (from Jablonka & Raz, 2009).

We are concentrating on the route of between-generation transmission that involves a single-cell "bottleneck", transmission through a gamete or a spore in multicellular, sexually reproducing organisms, or through a single sexual or asexual cell in unicellular organisms. The environment may induce epigenetic variation by directly affecting the germline or by affecting germ cells through the mediation of the soma, but, in either case, subsequent transmission is through the germline. The direct soma-to-soma transmission route of epigenetic variations is of great importance for both development and evolution (Jablonka & Lamb 2005, 2007a,b).

The organization of chromatin and chromosomes, their localization in the nucleus, and the dynamic interactions among the various components of chromatin determine when, where, and to what extent genes are transcribed, how DNA repair is orchestrated, how different chromosomal domains are organized, and how chromosomes, as units, behave during the various phases of the cell cycle. Patterns of chromatin are reconstructed following DNA transcription and replication, and, although the processes of reconstruction are not well understood, there is evidence that chromatin variations can be transmitted between generations of individuals. The study of the chromatin-marking EIS (epigenetic inheritance system) is therefore crucial for the understanding of both development and heredity. Methyl groups are covalently attached to DNA and bound histone and nonhistone proteins, and associated RNA molecules. DNA methylation, the best-understood system of chromatin inheritance, is an epigenetic modification found in Eubacteria, Archeabacteria, and Eukaryota and it is involved in many important functions, such as regulation and maintenance of gene activity patterns (Barlow & Bartolomei 2007) or DNA replication and repair (Mortusewicz et al. 2005; Schermelleh et al. 2007). Variations in a DNA methylation pattern can be inherited between generations. How a DNA methylation pattern affects a cell's or an organism's phenotype depends on the way it interacts with the protein components of the chromosome, which are also heritable. In eukaryotes, important variations in chromatin are associated with histones, the proteins that make up the nucleosome core around which DNA is wrapped. The dynamic nature of histones, their variability, and their association with every conceivable cellular function (see Berger 2007; Groth et al. 2007; Blasco 2007; Morris and Moazed 2007) makes understanding the inheritance of their specific structure and organization urgent.

Unlike the replication of DNA variations, which is largely context insensitive, whether and for how long a particular mark or cellular element is transmitted between generations depends on

genomic, developmental, and external conditions. This does not mean that conditions that allow epigenetic inheritance are particularly rare. Variations in epigenetic marks can be inherited for several generations (Jablonka & Raz, 2009). Heritable epigenetic variations and epigenetic control mechanisms are relevant for the empirical and theoretical study of evolution because they affect both the processes of adaptation and of divergence (Jablonka & Lamb, 1995, 2005, 2006, 2007b). Adaptation can occur through the selection of heritable epialleles, without any genetic change.

Epigenetic variants are often induced when environmental conditions change, so several individuals in the population may acquire similar modifications at the same time. This means that adaptation through the inheritance of newly induced epigenetic variants may be very rapid (Jablonka & Lamb 1995, 2005; Kussell and Leibler 2005; Richards 2006; Bossdorf et al. 2008), thus leading to the accumulation of epigenetic variations. The relevance of epigenetic variations to biodiversity in our rapidly changing world is of obvious interest and clearly has to be further investigated (Jaclonka & Raz, 2009).

There are many paths that epigenetic inheritance can take, of course, but one of them, especially in species with extended infancies or juvenile periods, is via maternal effects (Bjorklund, 2006). Maternal effects are defined as the influence of the maternally provided environment on the phenotype of her offspring (Legates, 1972) and their adaptive significance has been increasingly recognized. Maternal effects occur when a mother's phenotype influences her offspring's phenotype independently of the female's genetic contributions to her offspring. Many maternal effects can be modeled as environmentally modulated transgenerational phenotypic plasticity, in which environmental variation (e.g. temperature) experienced by mothers is translated into phenotypic variation in offspring. Maternal effects are no longer relegated as simple 'troublesome sources of environmental resemblance' that confound our ability to estimate accurately the genetic basis of

traits of interest. Rather, many maternal effects have been shaped by the action of natural selection to act as a mechanism for adaptive phenotypic response to environmental heterogeneity. Consequently, maternal experience is translated into variation in offspring fitness (Mousseau & Fox, 1998).

The idea that epigenetic variance could create phenotypic differences between individuals in the absence of genetic changes positions epigenetics as a possible mechanism for non-genetic transmission (Szyf, 2015).

Epigenetic theories of evolution view a developing organism's response to environmental changes as a mechanism for phylogenetic change. Natural selection still plays an important role in evolution, but it is the developmental plasticity of an organism that provides the creative force for evolution (Gottlieb, 1987, 2002; Lickliter & Schneider, in press; West-Eberhard, 2003).

1.2.2 Non-genetic inheritance

Natural selection is highly inefficient and slow in responding to immediate environmental challenges. It is well known that physiological systems can respond and adapt to new changes in real time, but the question remains whether there are non-genetic processes that could establish stable phenotypes and whether these can be inherited through germline transmission across generations. Biological examples have been documented of phenotypic plasticity emerging in relatively fast time-scales, and of frequencies that are orders of magnitude higher than can be explained by natural selection. Real transgenerational non-genetic inheritance could potentially result in a stable new trait. Therefore, a provocative question is whether such mechanisms might play a role in 'rapid evolution' of traits in response to new experiences and environments at rates that are orders of magnitude

faster than natural selection of stochastically arising genetic alterations (Szyf, 2015).

DNA methylation and hydroxymethylation are part of the covalent structure of DNA and are therefore the most proximal and certain epigenetic mechanisms. Thus, the DNA molecule itself contains both genetic and epigenetic information.

One of the interesting properties of DNA methylation is its heritability, and the fact that it can be replicated places it as an excellent candidate for serving as a transgenerational epigenetic mark. It was long believed that DNA methylation, although faithfully conserved in differentiated cells, was completely erased in primordial germ cells as well as shortly after fertilization and conception. If the erasure of DNA methylation was complete, then transgenerational transmission through DNA methylation marks would be impossible. The idea that DNA methylation is erased in the gametes during primordial germ cell differentiation was an important reason for the initial rejection of the idea of transgenerational transmission of DNA methylation marks in sperm. However, there is evidence from animal models for persistent DNA methylation changes in sperm that are transgenerational. It is now clear that, although the erasure of DNA methylation during primordial germ cell differentiation and post fertilization is fairly extensive, it is not complete.

Compare to migration out of stressful zones, or within-generation phenotypically plastic responses, or evolutionary changes, the mechanism of non-genetic inheritance has received less attention.

A large range of processes such as transmission of epigenetic variation, hormones, nutrients, parental glandular secretions or behaviors to offspring are comprised by non-genetic inheritance, which also encompasses maternal, paternal and parent-offspring indirect genetic effects and can be considered an extension of trangenerational plasticity (TGP).

Rapid transgenerational acclimation to temperatures expected by 2050-2100, with full restoration of aerobic capacity, was observed in tropical damselfish *Acanthochromis polyacanthus* (Donelson et al. 2012) and could allow species to persist across their present locations. Given this ability to maintain aerobic capacity and acclimate, we would also expect performance in growth and swimming ability at increased temperatures to be maintained. The transgenerational acclimation to elevated temperature is most likely explained by non-genetic parental effects or epigenetic inheritance. The phenotypic differences thus do not originate from variations in DNA base sequences (Jablonka et al. 2009).

Environments' transgenerational effects, which are mediated by non-genetic inheritance, are likely to affect direction and rates of adaptation to changing environments. This theory has led to focus on two questions: whether a population's mean phenotype, thanks to non-genetic inheritance, is able to track environments that are changing too rapidly for genetic adaptation and, always considering changing environments, how selection acts on non-genetic inheritance.

On the one hand, when environmental fluctuations are very rapid and unpredictable, non-genetic inheritance can be disadvantageous, because the environment experienced by the parental generation may be different to that of the future offspring and the non-genetic ingeritance can end up being maladaptive (Paenke et al. 2007). On the other hand, if the environment is changing in a predictable way, for example in the context of global warming, non-genetic inheritance as a form of adaptive TGP, can be beneficial and parents will advantageously produce offspring with optimized phenotypes for the anticipated conditions. In populations facing a long-term environmental trend like increasing temperature, we predict that selection will increase the frequency of non-genetically transferred traits that are advantageous, leading to adaptation of the population-mean phenotype over several generations and thus allowing a response to selection even without genetic variation.

Moreover, increasing populations' fitness, non-genetic inheritance is able to facilitate shifts into new niches, so populations that are in new environments have a mean phenotype that can potentially change over multiple generations thanks to these non-genetic mechanisms that mediate the transmission of immediately or at least potentially adaptive phenotypic traits.

There are lingering doubts about whether stable transgenerational effects are truly epigenetic, or whether they are genetic differences that are misconstrued as non-genetic inheritance.

The results of the study conducted by Verhoeven et al. (Verhoeven et al. 2010) show that environmental stresses readily induce DNA methylation changes at a genome-wide scale and demonstrate that most of the induced changes are faithfully transmitted to offspring. These results reflect transgenerational epigenetic plasticity of a single genotype in response to environmental stress.

1.2.3 Transgenerational Plasticity

Major climate changes are expected in the near future and, assuming that species' optimal climatic conditions do not change, these climate shifts may cause extinctions in a wide range of taxa. Because temperature has a dominant role in shaping metabolism, lifespans, and population dynamics, a mechanistic understanding of responses to changing temperature is needed, particularly for ectotherms. To date, attempts to address this issue have focused exclusively on evolutionary change and phenotypic plasticity. I highlight a third potential mechanism for rapid responses to climate shifts: transgenerational plasticity (TGP).

TGP, a generalization of more widely studied maternal effects, occurs when the environment experienced by one or both the parents prior to fertilization directly translates, without alteration of DNA sequence, into changes in offspring reaction norms, that modify the interaction between

parental and offspring environment effects. Where the mother's environment drives the response, TGP may be thought of as a type of maternal effect. Because paternally transmitted environmental effects also exist, 'transgenerational plasticity' is adopted to include the role of either parental environment in shaping the reaction norm of the offspring.

Transgenerational plasticity is a taxonomically widespread mechanism that responds to environmental drivers impacted by climate change. Evidence for non-genetic transgenerational inheritance spans the tree of life, including plants (Lau et al. 2008) and fruit flies (Gilchrist et al. 2001), and includes examples of parents' experiences increasing the tolerances of offspring to such environmental perturbations as contaminants (Marshall 2008), food shortages (Bashey, 2006), desiccation (Yoder et al. 2006) and shading (Galloway & Etterson, 2007). For many ectotherms, temperature may be the most relevant climate driver, since variation in temperature is important in most aspects of ectotherm biology, yet very little is known about transgenerational effects on thermal physiology.

In addition to temperature, other environmental variables taken into consideration in studies reporting evidence of TGP are drought stress, relative humidity, CO_2 concentration, salinity stress, light level, contaminant exposure, hypoxia and food availability. However, the most common variable used in TGP studies is temperature, which makes sense considering that periods of relevant temporal autocorrelation are present with thermal regimes and many populations display a seasonal phenology in reproduction timing. When TGP is suspected, a good indicator for deducing the predictability of the parent-offspring environment can be temperature time series data.

Before providing evidence for TGP in sheepshead minnows, transgenerational effects of changes in temperature had been studied in plants such as *Arabidopsis thaliana*, to which warm temperatures in the parental environment conferred competitive advantages. These plants were

grown in warm temperatures and produced seeds that showed a higher content of nitrogen and, consequently, the offspring exhibited increased biomass, seed production, and germination rates (Blonder et al., 2007). Therefore, non-genetic inheritance may help these plant populations succeed with global warming. There are different phenotypic effects of temperature experienced by parents on offspring also in *Drosophila*. A linear increase of *D. melanogaster* offspring's fitness was observed with temperature experienced by parents increasing from 18 to 29°C, regardless of the temperature the offspring experienced. Evidence for TGP was already provided in response to different environmental variables, for example higher CO_2 concentrations change TGP responses induced by predators in aphids (Mondor et al. 2004) and elicit TGP responses in three plant species (Lau et al. 2008). Therefore, alterations in growth represent a minor part of a very wide range of transgenerational responses to climate change. These empirical examples show how phenotype and fitness of descendants can be affected, potentially for multiple generations, by changes in temperature and other environmental variables.

TGP affecting lethal temperatures has been found in several species, but thermal TGP affecting a key life history trait such as growth, had been detected in only three species: a plant (Blödner et al. 2007; Whittle et al. 2009) and two insects (Groeters and Dingle 1988; Steigenga and Fischer 2007). Knowing whether ectothermic vertebrates have the capacity for thermal TGP is important when forecasting species' responses to temperature fluctuations and, if present, thermal TGP could affect a number of size-related characters (e.g. mortality, predation, movement).

1.3 SHEEPSHEAD MINNOWS

The first evidence for thermal TGP in a vertebrate life history comes from sheepshead minnows, *Cyprinodon variegatus* (Salinas & Munch, 2012). These are small fish (Fig. 4) common to nearshore marine and estuarine waters throughout the USA east coast and the Caribbean; they mature in approximately 2–3 months, live up to 3 years, breed nearly continuously under laboratory conditions and are an integral part of estuarine food webs.

Fig. 4. Sheepshead minnow (*Cyprinodn variegatus*).

Their distinguishing characteristics include silver bodies, single dorsal and anal fins, and no lateral line. The male is generally larger and deeper-bodied than the female, with larger dorsal, pelvic and anal fins. They reach sexual maturity at approximately 2–3 months. In colder water, they spawn from February through October; in warmer waters, spawning can occur throughout the year. Females can spawn several times during the spawning season at 1-7 day intervals, depositing between 100 to 300 eggs per spawning event. Eggs hatch after 3 to 12 days, depending on water temperature. They live up to 3 years, breed nearly continuously under laboratory conditions and are an integral part of

estuarine food webs. Sheepshead minnows are among the most eurythermal of all fishes, tolerating a large range of temperatures. Sheepshead minnow growth appears to be phenotypically plastic with respect to temperature along the U.S. east coast (Berry, 1987). Sheepshead minnows have been observed at temperatures as high as 43°C during summer (Harrington and Harrington, 1961) and are known to endure severe cold fronts that can reduce water temperatures from 15°C to freezing (-1.9°C) in less than 24 h (Moore, 1976; Bennett & Judd, 1992b). Sheepshead minnows are amongst the most eurythermic of all known fishes. This species' physiological and ecological thermal tolerance polygons (Fig. 5) have areas which are among the largest ever measured for a fish.

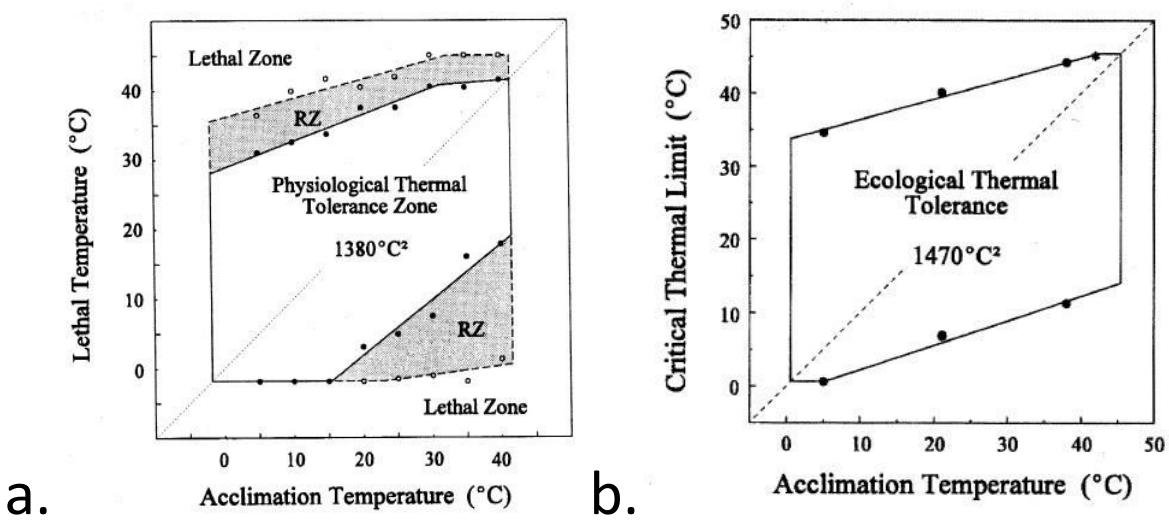

Fig. 5. **a:** Physiological thermal tolerance polygon for sheepshead minnow determined from upper and lower incipient lethal temperatures (solid circles). The resistance zones (RZ) are defined by upper and lower lethal temperatures (open circles), and areas outside the polygon represent lethal temperatures.

b: Ecological thermal tolerance polygon for sheepshead minnows. Area of the ecological tolerance zone was determined from critical thermal minima and maxima (solid circles) across the range of possible acclimation temperatures (From Bennett et al. 1997).

Not only are sheepshead minnows characterized by unparalleled physiological tolerance, they are more resistant to thermal extremes than other fishes. Due to their broad ecological and physiological thermal tolerance limits, sheepshead minnows are able to survive temperature extremes in their natural environment that nearly span the vertebrate biokinetic zone. Their exceptional thermal resistance provides short-term protection from intense midday summer extremes as well as low lethal temperatures during cold fronts (Bennett et al. 1997).

When given sufficient time, Sheepshead minnows beneficially program their offspring for maximal growth at the temperature the parents are in before fertilization of the eggs (Salinas & Munch, 2012).

In this previous study, Sheepshead minnows were raised for a generation in the laboratory at 21-22°C and were then transferred to either 24, 29 or 34°C. After exposure times of 7 and 30 days, eggs were collected, briefly after females spawned (fertilized eggs were exposed to the parents' temperature for less than 2 hours), and they were divided into the three experimental temperatures. Juveniles' growth in length was measured over 4-6 weeks and the growth rates obtained show how the offspring reaction norms depended upon the temperature that the parent experienced. After only 7 days of parental exposure there was no significant interaction between parent and offspring temperature, but after 30 days of exposure of the parents to the experimental temperatures, this interaction was evident, showing how there was a shift in reaction norms of juveniles driven by the temperature that precedes fertilization (Fig. 6). Sheepshead minnow parents therefore alter the response to temperatures in their offspring, and higher growth rates in early stages and therefore larger body sizes also mean increased survival and fecundity in fishes.

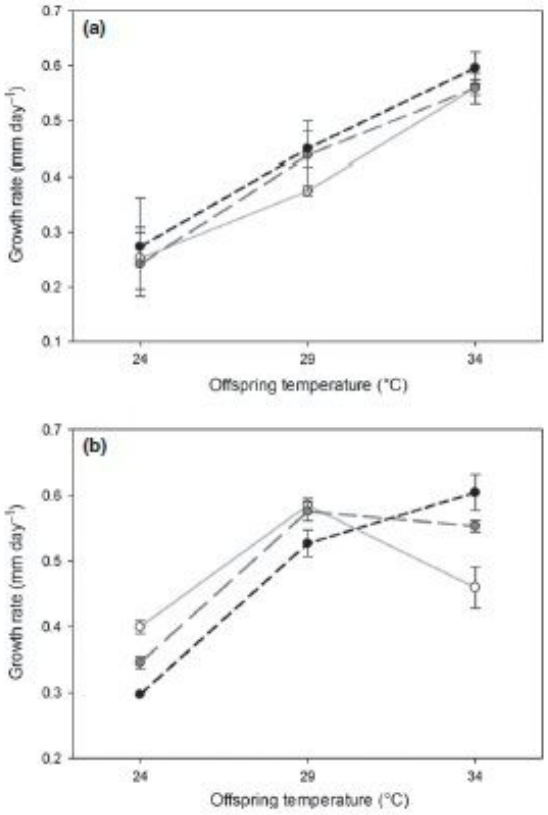

Fig. 6. (a) Offspring growth vs. temperature for parents held for 7 days at 24, 29, or 34 °C (white, gray and black circles respectively). Growth is parallel across parent temperatures, i.e. there is no effect of parent temperature on growth (P = 0.73).

(b) Offspring growth vs. temperature for parents held for 30 days at 24, 29 or 34 °C (white, grey and black circles respectively). The interaction between offspring and parent temperatures is significant (P < 0.001). (From Salinas & Munch, 2012)

Sheepshead minnows are ectothermal organisms and therefore they present a dome-shaped temperature-growth rate profile (Fig. 7).

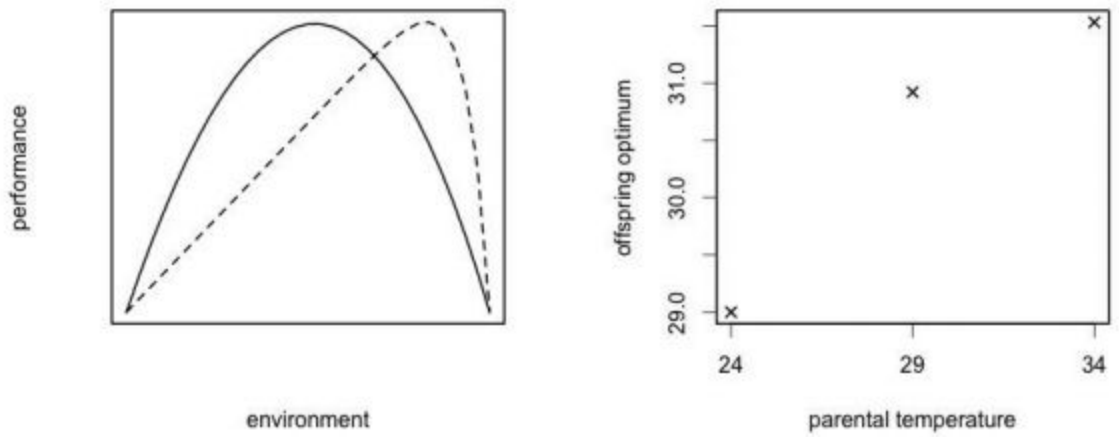

Fig. 7. (a) In ectotherms, the temperature (x-axis)-growth rate (y-axis) relationship is a domed function. TGP is manifested by a shift in the offspring relationship based on the experience of the parent. (b) The temperature for optimal growth of sheepshead minnows depends upon the temperature that their mother experienced. (From Salinas et al. 2013)

Thermal performance curves tend to have the same shape with performance increasing with rising temperature, reaching a maximum (the temperature optimum T_{opt}) at an intermediate temperature before re-decreasing. Phenotypic plasticity can shift the curve vertically and horizontally and can alter the position of the T_{opt}.

The interest in these curves has increased due to their potential utility in contributing to the prediction of species or population responses to climate change. They can in fact be incorporated in models of organism responses to changing climate, potentially beneficial for predicting climate change's effects on the persistence of species.

In populations facing a long-term environmental trend like increasing water temperature due to global warming, we predict that selection will increase the frequency of non-genetically transferred

traits that are advantageous, leading to adaptation of the population-mean phenotype over several generations and thus allowing a response to selection even without genetic variation. Therefore, the innovative idea of populations being able to adapt to rapidly shifting environments, through non-genetic mechanisms such as transgenerational plasticity, opens new possibilities of survival of species.

2. PURPOSE OF RESEARCH

As climate change occurs at global and more local scales, biologists are trying to predict how the ecosystems and organisms are going to respond and many focus primarily on the responses of organisms to thermal variations (Sears et al. 2011).

Transgenerational plasticity (TGP), a generalization of more widely studied maternal effects, occurs when the environment experienced by one or both the parents prior to fertilization directly translates, without alteration of DNA sequence, into changes in offspring reaction norms. Such effects have been observed in several traits throughout many phyla, but, despite enormous potential importance, especially with rapid climate change, TGP in thermal growth physiology had never been demonstrated for vertebrates.

Provided the first evidence for thermal TGP in sheepshead minnows in the Salinas & Munch's experiment (Salinas & Munch, 2012), my project seeks evidence of life history TGP in sheepshead minnow eggs and larvae whose parents were sampled from Connecticut and South Carolina and exposed to these different temperatures.

In order to estimate impacts of climate change on marine organisms, the approach utilized is usually to sample organisms from the field, expose them to some predicted future change, for example in temperature or acidity, and then test for the exposure's impact on some metric of performance (Byrne, 2011). My study exposed sheepshead minnows to different temperatures and I quantified parents' egg production, egg sizes, hatching rates, the dimensions of larvae at hatching and after one week to calculate the larval growth rates during their first week after hatching, and the larval survival rates.

I investigated whether egg sizes and egg numbers depended on maternal temperature and whether these differed among populations, and I wanted to establish whether they changed with the duration of the parent exposure period to the experimental temperatures. My aim was also to assess whether exposure time, parent temperature, offspring temperature or the interaction between parent and offspring temperatures had significant effects on egg viability and larval survival rate. Lastly, I wanted to test if there was evidence of TGP in the one-week larval growth rates and, if so, how the degree of TGP (i.e. size of the temperature interaction term) changed with the 5, 12, 19, 26, 33 and 43 day exposure times, which is considering a greater resolution than the 7, 30, 45 day exposure times of the previous study (Salinas et al. 2012).

3. MATERIALS AND METHODS

3.1 LOCATIONS

This experiment took place at the National Marine Fisheries Service laboratories, on the UC Santa Cruz coastal science campus (location 3 in Fig. 8). The sheepshead minnows were located here after being collected in two sites: the first in South Carolina (32°45'2" N 79°53'50" W, location 1 in Fig. 8), in July 2014, and the second in Connecticut (41°20'9.70'' N 72° 2'3.14" W, location 2 in Fig. 8), in September 2014.

Fig. 8. Locations of sampling sites and of where the experiment was conducted.

1: South Carolina site, where sheepshead minnows were collected in July 2014.

2: Connecticut site, where sheepshead minnows were collected in September 2014.

3: Location of the NMFS laboratories in Santa Cruz, California, where the fish were relocated.

3.2 EXPERIMENTAL DESIGN

This study consists of a nested experimental design. The factors considered are: populations (random factor with 2 levels: South Carolina and Connecticut, the latter considered only in analyses regarding egg counts and dimensions), parent temperature (fixed effect with 2 levels: 26°C and 32°C), exposure time (random effect with 6 levels: 5, 12, 19, 26, 33 and 43 days, of which the first level is not considered in larval growth rate analysis) and egg and larvae temperature (fixed effect with 2 levels: 26°C and 32°C). Also, the experimental design is unbalanced, with 10 replicates from South Carolina exposed to 26°C, 7 replicates from South Carolina exposed to 32°C, 6 replicates from Connecticut exposed to 26°C and 5 replicates from Connecticut exposed to 32°C.

3.3 TEMPERATURE INCREASE

The parental generations of this experiment were kept in aquaria at 24°C until the start of the experiment. After taking photos of all the adults (with a 10.1-megapixel EOS 40D Canon camera) and measuring all maternal sizes, using Image-J, a Java-based image processing program (Fig. 9), I randomly placed 5 pairs of adults (male and female) from the Connecticut population and 7 pairs from the South Carolina population into each of two sea tables at 24°C.

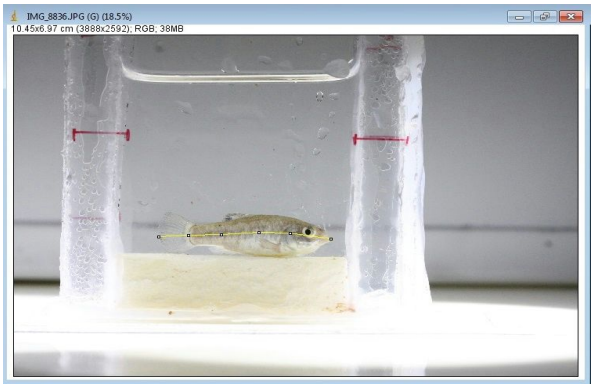

Fig. 9. Measurement of one of the mothers of the parental generation using Image-J.

Each pair was placed in a net breeder (BOYU net breeder for aquarium, model NB-3202A, in Fig. 10) and male and female were then separated by a plastic barrier that divided the net breeder in two halves, one per fish.

Fig. 10. Adult sheepshead minnows in net breeder before inserting the barrier to separate them in two different sides.

Over the next 5 days, I gradually raised the temperature in one tank to 26°C and the other to 32°C. The experimental temperatures were reached on April 20th 2015, which was defined as day 1 for gathering data.

3.4 FISH CARE

Feeding and daily care followed standard protocols (Cripe et al. 2009). Before and during the experiment, parents were fed every day with TetraMin flakes (Fig. 11 a) at 9:30 am and 4:30 pm, and frozen brine shrimp diluted in water (Fig. 11 b) at 1:30 pm.

Fig. 11. TetraMin flakes (**a**) and frozen brine shrimp diluted in water (**b**) fed daily to the parental generations.

The larvae that hatched during the experiment were maintained for a week and fed live rotifers, specifically 1000 rotifers per net breeder, so 9-12 ml (more than 1000 rotifers considering that some manage to escape through the net before the larvae reach them) of seawater containing rotifers at a concentration of 115 rotifers/ml of water, at 9:30 am and again at 4:30 pm daily.

Throughout the experiment, pH and levels of nitrite, nitrate and ammonia were monitored in the tanks containing the parents, eggs and larvae. Also 10% of the water in every parental tank was replaced daily, draining every table for 2 minutes then refilling with either reservoir sea water or a combination of reservoir sea water and freshwater, to maintain a constant salinity of 20 ppt. Eggs and larvae were placed in artificial sea water (freshwater and artificial sea salt).

3.5 EGG COLLECTIONS

To detect any influence of duration on transgenerational effects on the offspring, I collected eggs 6 times: on days 5, 12, 19, 26, 33 and 43. On these days, I introduced the egg-collecting mats (in the BOYU model NB-3202A set) into the net breeders and placed them on the bottom of the

container at 5:00 am, before the lights turned on, and I removed the barriers separating male and female in the net breeders. Fertilized eggs remained at parental temperatures for < 3 hours prior to collection, starting at 8:00 am (Fig. 12).

Fig. 12. Several stations equipped with Petri dishes, tweezers, methylene blue, artificial sea-water, everything that is necessary for the egg collection and transfer into the new 26°C or 32°C tanks.

Eggs from each parental temperature were collected from the mats, disinfected in methylene blue, pooled and then subdivided in two groups before transfer to smaller nets (MARINA fish net breeders, Fig. 13) in tanks at either 26°C or 32°C.

Fig. 13. MARINA fish net breeder in which eggs were placed after egg collections and in which also the larvae were kept.

Upon collection, we counted and photographed the eggs (Fig. 14) in order to measure the egg diameters using Image-J. Photographs were taken with a 10.1-megapixel EOS 40D Canon camera and a macro lens mounted on a tripod (calibration photos were taken at the beginning of each session and the tripod never moved within sessions).

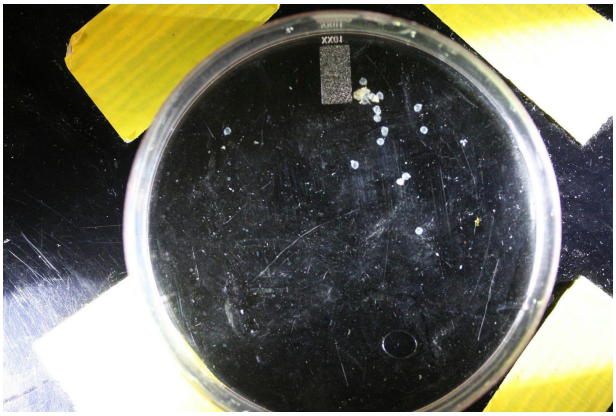

Fig. 14. Example of a photograph (taken with a Canon 40D) of one of the Petri dishes containing half of the eggs collected from one of the egg mats (the other half in another Petri dish was destined to another temperature).

3.6 LARVAE MEASUREMENTS

The viable eggs hatched 2-10 days after egg collections, the larvae were counted in order to obtain hatching rates, and photographed in order to measure (with Image-J) the larvae's lengths at hatch. Starting with the day 12 egg collection, larvae were kept in MARINA fish net breeders in separate tanks (but at the same temperatures) for a week and then re-photographed (always using the EOS 40D Canon camera with macro lens mounted on a tripod and calibration photos taken at the beginning of each session, with tripod never being moved within sessions) in order to obtain one-week measurements with Image-J (Fig. 15).

Fig. 15. Example of a photograph (taken with a Canon 40D) of one-week-old larvae.

A week after hatching, the larvae were photographed and then euthanized using TRICAINE-S (Brand of Tricane Methanesulfonate, MS-222).

I calculated larval survival with the larval counts at hatch and before euthanization, obtained larval growth rates from the larval sizes at hatch and after 1 week, and calculated hatching rates from count of fertilized eggs and eggs hatched.

3.7 ANALYSES

Statistics were done in the R computing environment, using ANCOVA and one-way ANOVA mixed models. The analyses in which effects were proved to be significant were followed by a Tukey Kramer multiple comparison post hoc test. Statistical differences were accepted for values with $P<0,05$. Graphs were made using RStudio and Excel.

4. RESULTS

4.1 ANALYSES ON EGG DATA

Firstly, an ANCOVA was performed on the total egg diameter and egg number averages, considering all the exposure times together. Maternal size was treated as a covariate and I tested the effects of two factors: parent temperature and population. These analyses showed a significant effect, confirmed by the post hoc Tukey Kramer procedure, of the parent temperature (P=0,008) on the overall egg diameters (Fig.16). No significant effect of parent temperature or population on the total amounts of eggs produced was detected.

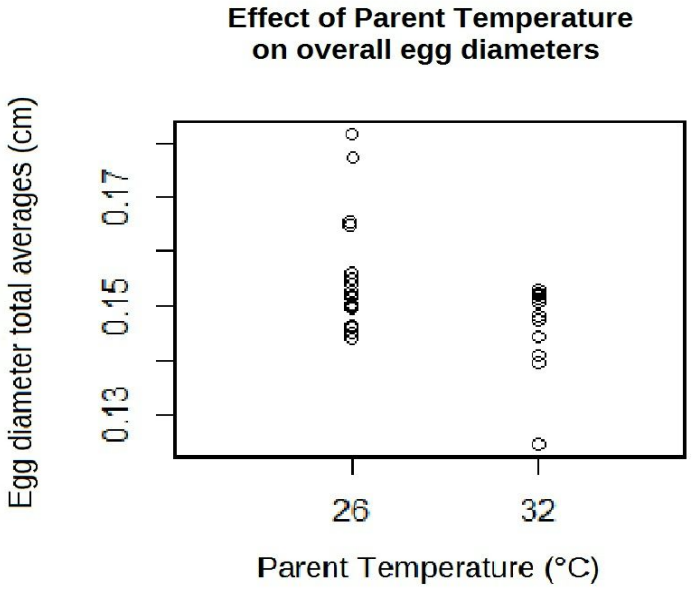

Fig. 16. R plot representing the significant (P<0,01) effect of Parent temperature on the total egg diameter averages.

Secondly, I performed ANCOVAs on egg sizes treating population (SC and C) and temperature (26°C and 32°C) as treatments and maternal size as a covariate, separately for eggs at exposure days 5, 19 and 33. The same ANCOVA was performed on the egg numbers with the same covariate and treatments, at the same exposure times. A covariate analysis showed a significant effect of maternal sizes (P=0,012) on the diameters of the eggs produced at the 5 day exposure period and 19days of parent exposure (Fig. 17). Moreover, in addition to the covariate being significant (P=0,03) there was a very significant effect of parent temperature (P=0,0006) on egg sizes at the 19-day exposure time (Fig. 18), confirmed by the post hoc Tukey Kramer test. No significant effects resulted from the ANCOVA on egg diameters for the 33-day exposure time. Also, for all exposure times there was no significant effect of population or parent temperature on the amount of eggs produced.

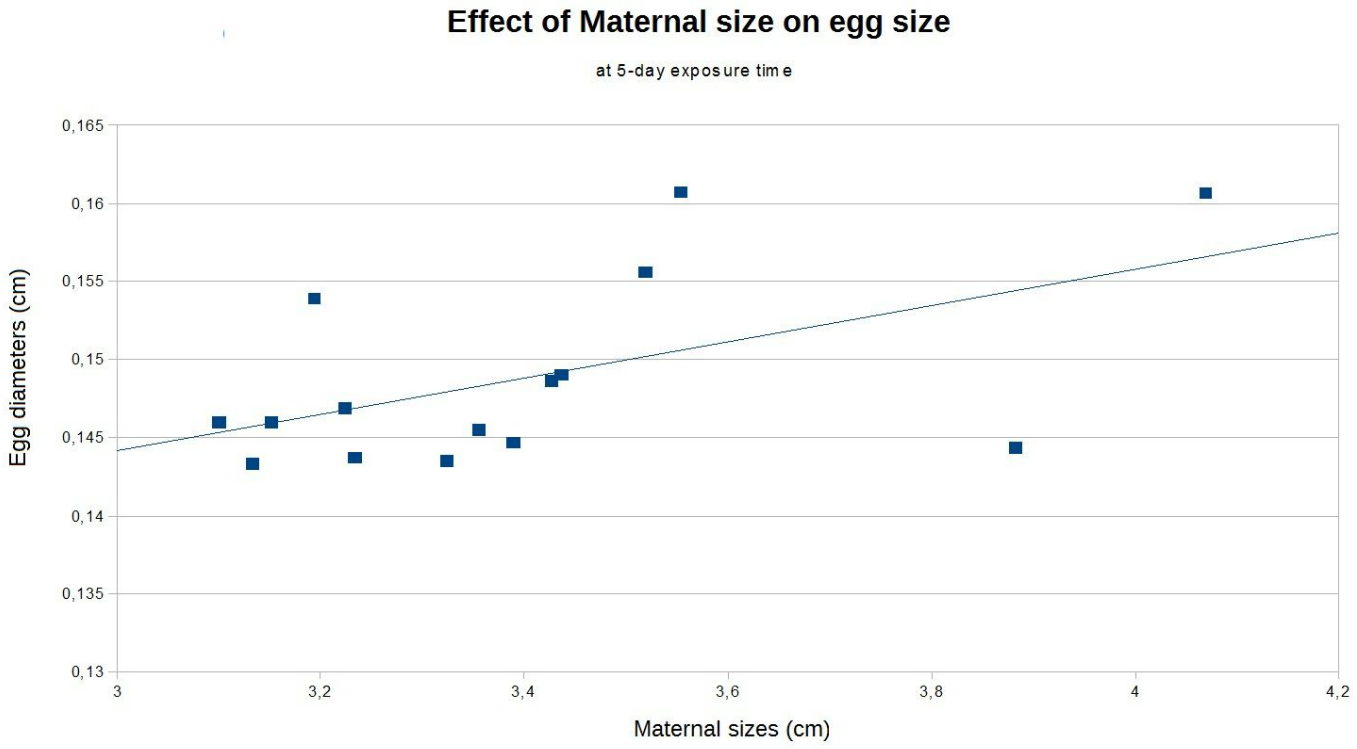

Fig. 17. Excel scatter chart representing the significant effect (P<0,05) of the covariate (maternal size) on the egg sizes, at the 5-day exposure time.

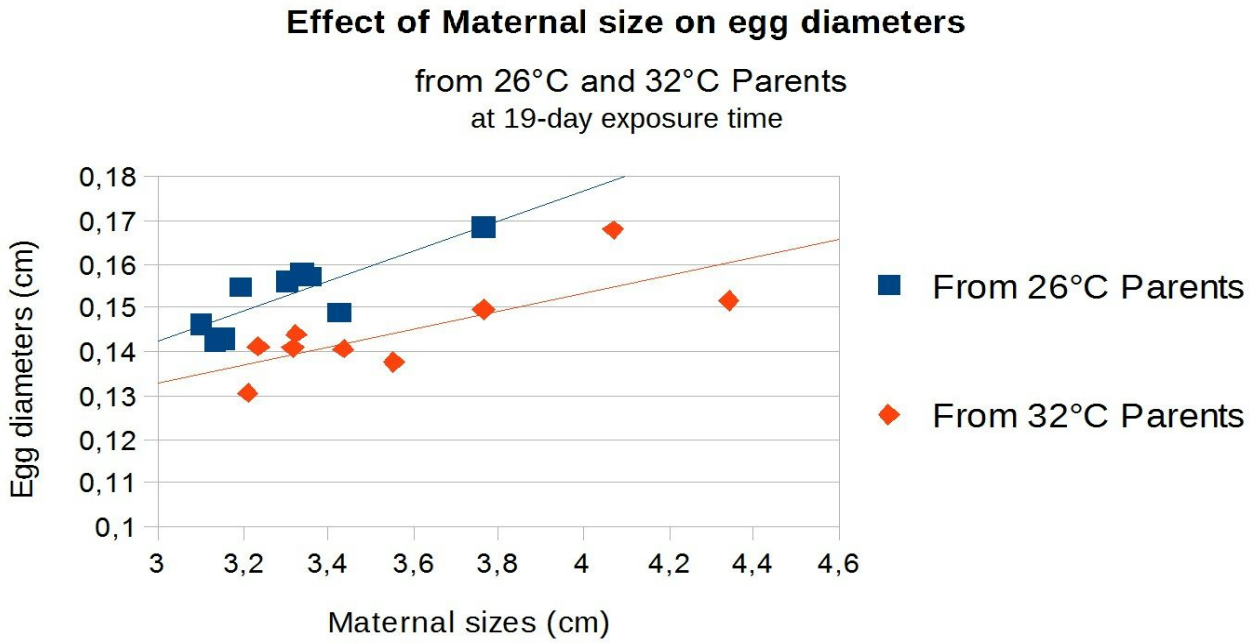

Fig. 18. Excel scatter chart representing the significant effects of maternal size (P<0.05) and parent temperature (P<0.001) on egg diameters at the 19-day exposure period.

Afterwards, I tested for an effect of the exposure duration on the egg diameters and numbers, for each population separately, with a 'repeated measures' ANOVA, to account for the fact that eggs are taken from the same family repeatedly. Family ID was treated as a random effect. There was no significant effect of the exposure duration on the egg sizes for either population. However, the effect of exposure time on the amount of eggs produced was very significant for both Connecticut (P=0,009) and South Carolina (P=0,009) populations (Fig.19-20). With the post hoc Tukey Kramer multiple comparison procedure I further investigated where the significant differences between egg number means were, in both populations.

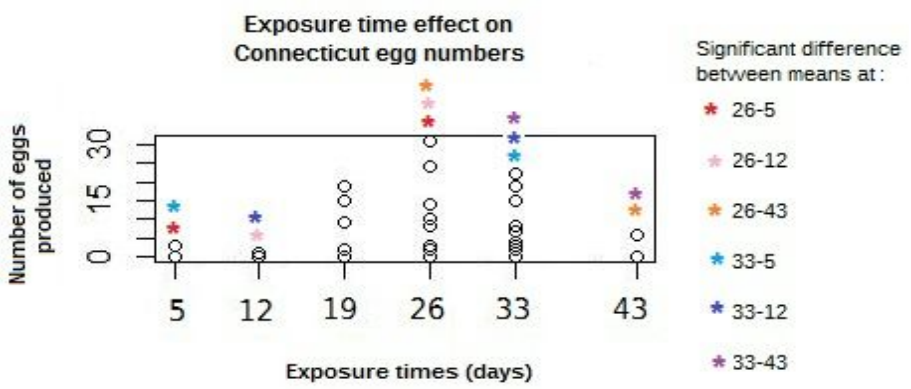

Fig. 19. R plot representing the significant effect (P<0,01) of the duration of the parent's exposure to the 26°C and 32°C temperatures on the amount of eggs produced in the Connecticut population. The Tukey Kramer multiple comparison post hoc test assessed that the egg number means at the 26-day and 33-day exposure times were significantly different from most of the other means. The specific significantly different pairs of means that resulted from this test are listed on the right side of the figure and indicated on the graph with colour-coded asterisks. The egg number mean at the 19-day exposure time was not significantly different from any of the other means.

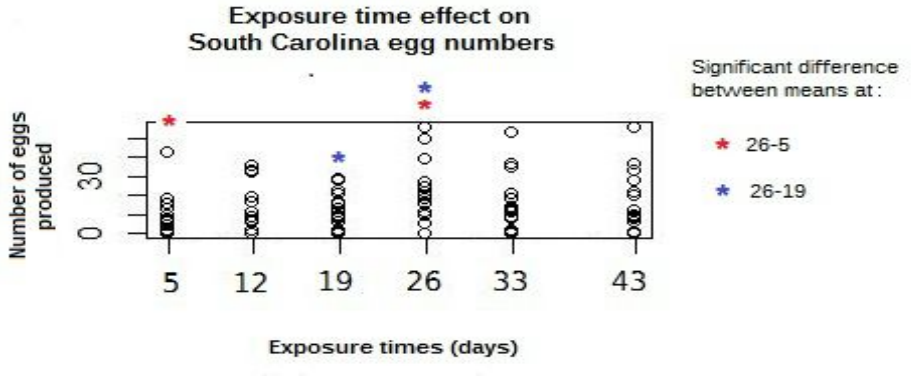

Fig. 20. R plot representing the significant effect (P<0,01) of the parent's exposure period to the 26°C and 32°C temperatures on the amount of eggs produced in the South Carolina population. The Tukey Kramer multiple comparison post hoc test assessed that the egg number mean at the 26-day exposure time is the most significantly different from other means. The specific significantly different pairs of means that resulted from this test are listed on the right side of the figure and indicated on the graph with colour-coded

asterisks.

4.2 LARVAE

The number of hatches from the Connecticut population was insufficient, there wasn't enough data to be informative, therefore in the following analyses I only considered the data concerning the South Carolina population.

"Repeated measures" ANCOVAs were performed on the percentages of hatch obtained from the initial larval counts, and on the percentages of larval survival, from the final larval counts after a week. Maternal sizes and egg sizes were taken into account as covariates and exposure time, parent temperature, offspring temperature, and the interaction between the two temperatures as treatments. These analyses showed an increasingly significant effect of exposure time (P=0.013, Fig. 21), parent temperature (P=0.0009, Fig. 22) and egg (offspring) temperature (P=6.08e-05, Fig. 23) on the percentage of hatch, while there was no significant effect on % of survival.

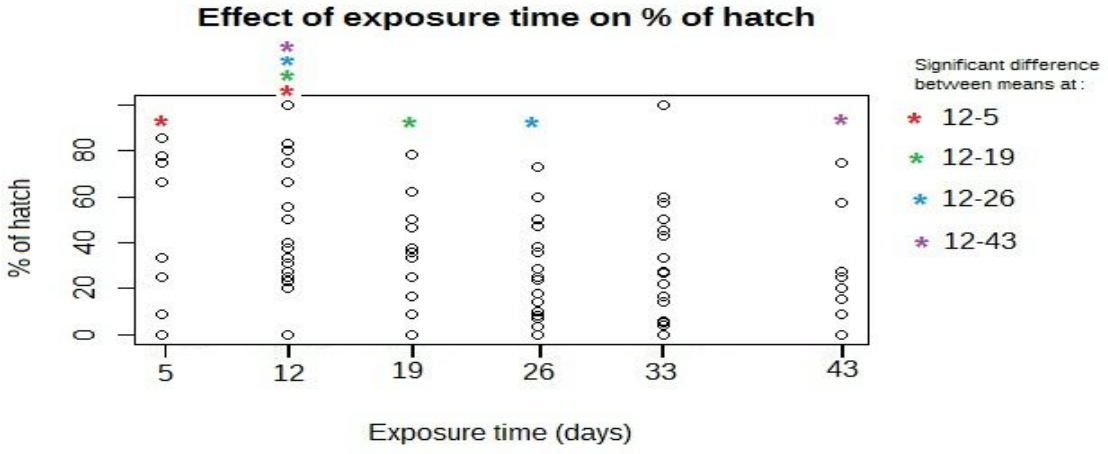

Fig. 21. R plot representing the significant effect (P<0,05) of the duration of the parent's exposure to the 26°C and 32°C temperatures on the percentage of hatch in the South Carolina population. The Tukey Kramer multiple comparison post hoc test assessed that the egg number means at the 12-day exposure times was significantly different from most of the other means. The specific significantly different pairs of means that resulted from this test are listed on the right side of the figure and indicated on the

graph with colour-coded asterisks.

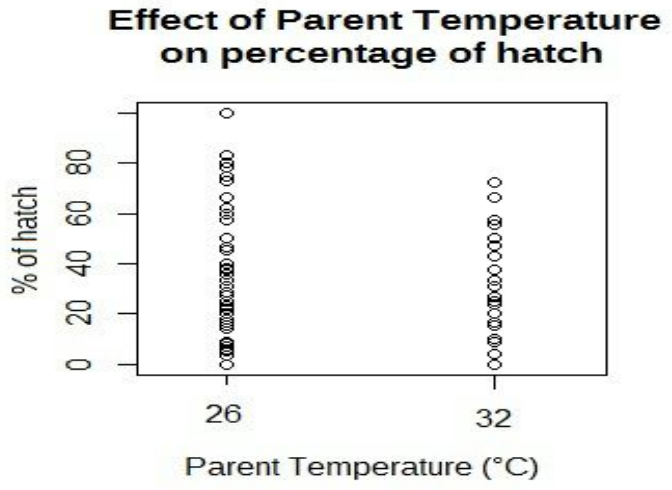

Fig. 22. R plot representing the significant effect (P<0,01) of parent temperature on percentages of hatch.

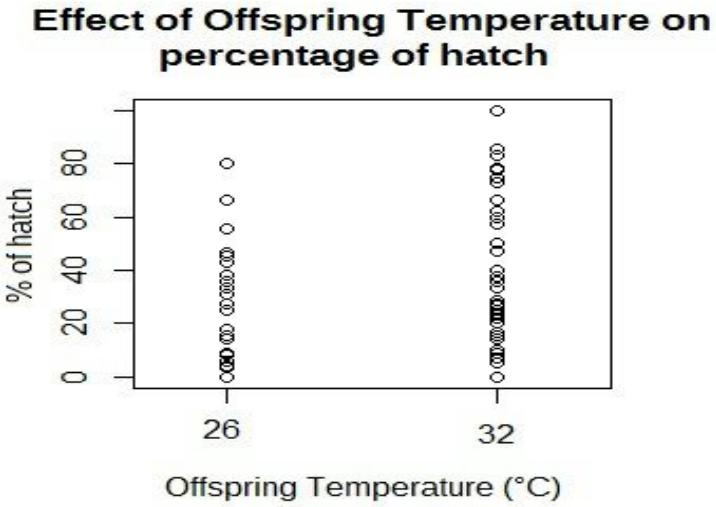

Fig. 23. R plot representing the significant effect (P<0,001) of offspring temperature on percentages of hatch.

Finally, an ANOVA was performed on the one-week South Carolina larval growth rates. I used egg sizes and maternal sizes as covariates and parent and offspring temperatures as treatments, also testing for parent temperature-offspring temperature interaction, separately for each parent

exposure time (12, 19, 26, 33 and 43 days). Offspring temperature effect on growth rates was significant at the 26-day (P=0,006) and 33-day (P=0,02) exposure times (Fig.24). However, no significant effect of parent temperature-offspring temperature interaction resulted from the analyses.

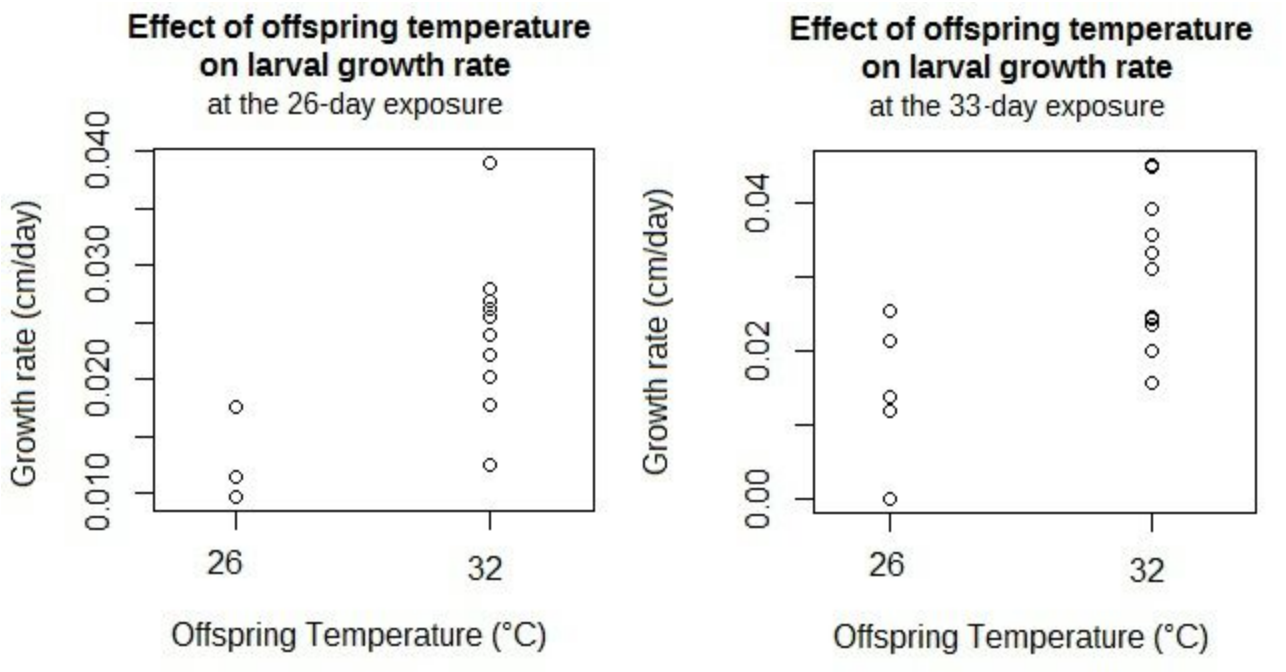

Fig. 24. R plot representing the significant effect of offspring temperature on larval growth rates at the 26-day (P<0,01) and 33-day (P<0,05) exposure times.

5. DISCUSSION AND CONCLUSIONS

5.1 INTERPRETATION OF THE RESULTS

5.1.1 Outcome of the egg analyses

What we wanted to investigate with the first ANCOVAs on egg diameters and egg numbers was whether egg size and egg number depend on maternal temperature and whether this differs among populations. Based on previous research on many other fish, higher temperatures were expected to lead to the production of smaller eggs. Also, I wasn't expecting a significant population effect, but it would have been interesting if the analyses had proved otherwise. These first analyses resulted, as expected, in a significant effect of the parent temperature on the overall egg diameters. Also the ANCOVAs on the diameters of the eggs produced at the single exposure times showed a very significant effect of parent temperature, in particular at the 19-day exposure time. There's a lot of evidence of this in the field, even looking for instance at egg sizes versus latitude or season. Egg sizes tend to be bigger during the winter than in summer. A possible explanation for this is due to the fact that diffusion is what limits oxygen into the middle of the egg, and this is a bigger problem when temperatures are high, firstly because the metabolism is higher and, secondly, because the oxygen saturation level of the water is lower. The parent temperature though did not have a significant effect at the 5-day exposure time, and this was also expected because the TGP experiment previously done on sheepshead minnows (Salinas et al. 2012) showed that there's still no TGP at less than 10 days and that the significant results are usually later, towards 30 days of exposure. We don't know exactly what happens between 10 and 30 days, yet. One of the reasons of doing this experiment was to see if I'd have the same results or if something new would come up. In fact the significance of the parent

temperature effect on the egg sizes at the 19-day exposure time represents new interesting information, and means that the fish are actually starting the parent effect earlier than 30 days.

A covariate analysis showed a significant effect of maternal sizes on the diameters of the eggs produced after most of the parental exposure times to the experimental temperatures. As expected, the larger mothers produced bigger eggs.

No significant effect of parent temperature or population was detected neither on the overall amounts of eggs produced nor on the numbers of eggs produced at the single exposure times. However, the effect of the parental exposure time to the experimental temperatures on the amount of eggs produced was very significant for both Connecticut and South Carolina populations. In particular, in the Connecticut population the egg number means at the 26-day and 33-day exposure times were significantly different from most of the other means, while in the South Carolina population the egg number mean at the 26-day exposure time was the most significantly different from the other means. In these last analyses testing the effect of exposure time on the egg data, the main idea was that I needed to account for the non-independence of egg sizes and numbers because they came from the same mother. My aim was to establish whether egg size and egg production changed with the exposure time. I recall that it was previously shown (Salinas et al. 2012) that the degree of TGP was 0 for exposures of 7-10 days, high at 30 days, and then leveled off or decreased at 45 days, depending on the population. Therefore I was expecting to see a drop in egg production between the second and fourth egg collection, corresponding to the shift in TGP. As a matter of fact, I did observe a significantly higher egg number mean around the 26-33 days exposure period and lower mean at the 12-19 days exposure period.

In the previous experiment, they found that around the 20-day exposure time, the parents were not producing many eggs, and that the eggs that they did produce had low viability. After the

20-day exposure period at which the mean is low, the egg production increased (consistent with my results) and percentage of hatch was also higher (discussed below). A possible hypothesis is that this may have to do with the time it takes the fish to adjust to the new experimental temperature after being at 24°C for last 6 months, and, concerning the fish from Connecticut, by the time they've "realized" they're at a higher temperature, their breeding season is almost over, therefore they shut back down before 45 days, which is the length of the Connecticut fish's breeding season. And this is consistent with the results I obtained that show a significant increase in egg production at 26 and 33 days and then a significant decrease by 43-day exposure time. In the previous experiment, the fish from South Carolina instead continue to produce eggs with TGP even at 45 days, which is consistent with the fact that the season for reproduction is a lot longer in South Carolina than in Connecticut. In my experiment the South Carolina egg production significantly increases at the 26-day exposure time, and, although only the mean at the 26-day exposure time resulted from the Tukey Kramer post-hoc test to be significantly different from the means of the previous exposure periods, we can see that the egg production remains high until the 43-day exposure time, and that absolute differences of the 33- day and 43-day exposure times from those before 26 days were very close to being significant.

5.1.2 Outcome of the larvae analyses

The ANCOVA on the percentages of hatch showed an increasingly significant effect of exposure time, parent temperature and egg temperature on the percentage of hatch.

Regarding the effect of the parental exposure time to the experimental temperatures on egg viability, the Tukey Kramer multiple comparison procedure showed a significant decline of the hatching rates towards the 19-day exposure time, consistent with the previous study. The means at the 33-day exposure period resulted not significantly different, however the absolute differences between the hatching rates at 33 days and the low values at 19 days, as we would expect from the

results of the previous experiment, was close to the critical range value. What was interesting was the significantly higher mean of the hatching rates at the 12-day exposure. However, this is only the second time someone does this kind of experiment, so it isn't surprising I got different answers. The hypotheses for the results of the previous experiment were that at the first egg collections, what we get are eggs that the fish had already been producing before the start of the experiment, because vitellogenesis takes up to 2 weeks in these fish, and that the significant decrease in egg production and viability around the 20-day exposure time was due to the fact that the adults were switching over to produce eggs that would be ready and better adapted for the temperature they're actually in.

Regarding the effects of Parent temperature and egg temperature on the percentages of hatch, I observed higher hatching rates of the eggs deriving from 26°C parents, and higher hatching rates in the eggs placed at 32°C after the egg collections. The fact that I had higher hatching rates from the eggs produced by the 26°C parents is consistent with the observation that I moved the fish to the experimental temperatures after months at 24°C, therefore especially the adults at 32°C (8°C higher) hadn't had the chance to acclimate, yet. Regarding the effect of the temperature in which the eggs were placed after the egg collections, we know that, compared to the eggs at 26°C, the eggs at 32°C tend to hatch in half the time (3-4 days vs. 7-10 days), therefore, if they die at a constant rate, a higher number of hatches at 32°C than at 26°C is consistent.

Lastly, I performed ANOVAs on the one-week South Carolina larval growth rates. The aim was to test how the degree of TGP (i.e. the size of the parent temperature-offspring temperature interaction term) varied, considering parent exposure times with a greater resolution than the 7/30/45-day exposure times we had from the previous experiment. The result we were hoping to observe was an increase and levelling off over the course of my experiment, but we also expected that there could have been more complicated outcome than that.

What resulted from these analyses was that offspring temperature had a significant effect on growth rates at the 26-day and 33-day exposure times. In both cases higher growth rates were observed at higher temperatures, which is consistent with the fact that at higher temperatures ectothermic organisms have higher metabolic rates and grow faster (Angilletta et al. 2004).

However, no significant effect of parent temperature-offspring temperature interaction resulted from the analyses, indicating no evidence of TGP in the growth rates.

A realistic explanation for the fact that I found no evidence of TGP that must be taken into consideration is that there was not enough data to be informative. Already the replicates I could start the experiment with were not numerous, moreover they didn't always produce eggs, a great part of the eggs produced did not hatch, and not all the larvae that I counted and measured at the beginning survived the week, leaving me with a significant amount of holes in my data set and not enough growth rates to combine the different parent temperature and offspring temperature interactions.

Furthermore, there's a lot of intrinsic variability in size, and, trying to measure differences in growth rates, what must also be considered is the possibility that they needed more time to actually show the differences. At the beginning, what we get are the differences in initial size, and it takes time for the differences in growth rates to accumulate enough so that you can see the differences in size due to the growth rate versus just the initial size variation.

Although I expected this as a possible outcome of the analyses on the growth rates in such limited time, we typically do see differences in the averages of the growth rates on the order of 20-30%, a big difference in growth rate, therefore it was not unreasonable to hope that with enough larvae, I would have been able to see a significant difference over the course of the first week. However, with small amounts of larvae, it is not surprising that no significant differences in growth rates were observed.

5.2 CONCLUSIONS AND FUTURE RESEARCH

This experiment turned out to be very interesting, I got results that are consistent with those obtained in the previous TGP study on sheepshead minnows (Salinas et al. 2012) and results that present new information. The story that is coming together is that there's the initial adjustment period to the new temperatures, a decrease in egg production, no signs of TGP for a first period (until the 26-day/33-day exposure period), and then the parents shift to producing eggs which are better adapted to the new temperature environment and we start seeing signs of TGP, and the duration of these periods depends on what population is considered and how long the breeding season of that population is.

There has been a lack of sufficient understanding of the capacity for marine organisms to adapt to rapid climate change and in particular global warming, so it is still unclear if we can actually predict in a reliable manner the impacts of climate change on marine ecosystems and populations. Both genetic adaptation and non-genetic phenotypic plasticity will be fundamental in adaptive response to future climate changing in the sea. Phenotypic plasticity helps populations persist by allowing them to respond adaptively to varying environments, it may help them persist in the short-term, buying time for genetic adaptation to progress in the long-term, and thus will probably be important in order for species with relatively long generation times to persist. Future research, however, must consider the potential for the conditions experienced by the previous generations to alter the inidividuals' response to changing environments.

Non-genetic inheritance is potentially important but still poorly understood as a factor in responses to rapid changes in the environment. Research in this area, that still needs to illuminate the scope, nature and significance of non-genetic inheritance in adaptation, is strongly motivated by the need to assess whether populations will be able to adapt rapidly enough to cope with new conditions

and avoid extinction. Thermal changes have altered the growth and distribution of many populations, and whether organisms can locate new areas better suited to their capacities, and have a shift in their range, determines whether the population considered is likely to go extinct.

The innovative idea of populations being able to adapt to rapidly shifting environments, through non-genetic mechanisms such as TGP, therefore opens new possibilities of survival of species, where moving to new sites with more optimal conditions isn't possible. Transgenerational effects on thermal performance are likely to have significant implications on physiology, ecology and evolution, alter the risk of extinction due to climate change, play a role in a number of size-related phenomena such as movement, mortality and predation, and generate complex population dynamics through time lags.

In determining TGP's role as organisms face climate change, there are still various questions that should be answered, for example how predictable the environment should be (considering that sheepshead minnows started showing transgenerational effects after 26-33 days but not at the previous exposure times), or what specific molecular mechanisms transduce the parents' environments into heritable epigenetic variation, or what amount of generation is necessary for the environment's non-genetic effects to be erased, whether there's a reduced response after a certain number of generations. Many questions remain.

TGP is extremely important and its mechanisms must continue to be further investigated because it will help predict how populations will react to impending changes in the environment, primarily global warming.

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