How predictable are the effects of environmental gradients on livebearing fishes (Poeciliidae)?

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Statement of Author (Francesco Santi's) contributions for each Chapter

Chapters 1 and 6:
I researched and wrote both of these chapters (with comments from my supervisor Dr. Rüdiger Riesch).

Chapter 2:
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Samples (fish) were originally collected by AC Petry and another scientist, Márcio Araújo. AC Petry, M Plath and R Riesch conceived the specific study objectives. I then performed data collection and analysis (including geometric morphometrics analyses, life-history dissections, and statistical analyses) and wrote the first draft of the manuscript; all co-authors contributed to the final version of the manuscript.

Chapter 3:
Authors: F Santi, R Riesch, J Baier, M Grote, S Hornung, H Jüngling, M Plath & J Jourdan
R Riesch, M Plath and J Jourdan conceived the original idea, and R Riesch, M Plath, J Jourdan and I designed different aspects of the presented study. J Jourdan, M Plath, S Hornung and R Riesch collected fish in 2013, while R Riesch and I collected fish in 2017. The common garden experiment was performed by J Jourdan and S Hornung, M Grote and J Baier conducted life-history dissections on fish caught in 2013 and those from the common garden experiment, while I performed life-history dissections on individuals caught in 2017. S Hornung and J Baier took pictures of fish caught in 2013 and those derived from the common garden experiment, Emily Vella and I took pictures of fish caught in 2013, and I set landmarks on all fish and conducted geometric morphometric analysis. H Jüngling ran population genetics. I then performed statistical analyses on phenotypic variation of all wild-caught individuals, R Riesch analysed the heritability of phenotypic variation between wild-caught and lab reared individual, and J Jourdan analysed the genetic structure of European mosquitofish populations. I wrote the first draft of the manuscript, and all authors contributed to the final version.

Chapter 4:
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M Plath conceived the original study idea, and J Gao, R Riesch, M Plath and I then designed the study. R Riesch and I sampled invasive *G. holbrooki* across Europe in 2017, while samples of invasive *G. affinis* across China were collected by J Gao, L Zhou, X Wang and M Plath. J Gao, L Zhou and I conducted dissections, DNA extractions, and PCR amplifications for multiple paternity analyses of *G. holbrooki* samples, while by J Gao, L Zhou, X Wang and M Plath conducted the same for *G. affinis* samples. GLMs were performed by J Gao and I, and path analysis was conducted by R Riesch. I wrote the first draft of the manuscript, and all authors contributed to the final version.

Chapter 5:
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I conceived and designed (with input by A Deacon and R Riesch) the study. I collected all guppies for the study in May 2018 with help by Research Technicians of the University of the West Indies at St Augustine. I then trained E Vella on life-history dissections and K Jeffress on geometric morphometrics, and then supervised their data collection. I performed all statistical analyses and wrote the first draft of the manuscript, and all authors contributed to the final version.

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Abstract

Environmental gradients are ubiquitous but when multiple gradients (inter)act, predicting organismal responses can be extremely challenging. In **Chapter 1**, I reviewed what we know about how environmental gradients affect biodiversity and our ability to predict organismal responses, focusing in particular on livebearing fishes (Poeciliidae). Poeciliids are a perfect model to study the predictability of the effects of diverse environmental gradients, owing to their extensive phenotypic variation and ability to cope with starkly different environmental conditions. In **Chapter 2**, I investigated the effects of multiple interacting environmental gradients on a small geographic scale on our ability to correctly predict phenotypic responses in *Phalloptychus januarius* from coastal lagoons in Brazil. I found that, while phenotypic responses were overall predictable, the multifarious selection experienced by these fish led to several unpredictable patterns, as some environmental factors had unexpected, strong influences on phenotypic diversification. In **Chapter 3**, on a much larger geographical scale, I found that both climate-dependent and -independent environmental gradients shaped phenotypic responses in invasive eastern mosquitofish *Gambusia holbrooki* from across Europe. Moreover, these responses were driven mostly by phenotypic plasticity rather than rapid evolutionary change. In **Chapter 4**, I analysed the effects of climate on multiple paternity levels in both invasive European *G. holbrooki* and invasive Chinese *Gambusia affinis*. I found that the predicted increased multiple paternity in colder climates was mainly due to indirect effects facilitated via life-history trait variation. Nevertheless this pattern potentially increases the invasive potential of these two species in each range. In **Chapter 5**, I found that considering a single, strong environmental gradient (here: oil pollution) does not necessarily increase predictability of phenotypic responses in guppies (*Poecilia reticulata*) from 11 populations in Trinidad. Lastly, in **Chapter 6**, I discuss my results highlighting still open research questions with respect to the predictability of diversification along environmental gradients.

Table of contents

Chapter 3: A century later: adaptive plasticity and rapid evolution contribute to geographic variation in invasive mosquitofish 108

List of figures

List of tables

Chapter 1: General introduction

Environmental gradients

Environmental variation shapes biological diversity worldwide at all levels of biological organization, from differences between populations to species distributions (Verberk 2011), to even promoting speciation (Doebeli and Dieckmann 2003). In 1807, following his voyages across South America, Alexander von Humboldt—widely recognised as the father of modern ecology—was among the first to describe how the relative abundance of plant species depended on the climatic conditions they were subject to: "*but although life is everywhere diffused, and although the organic forces are incessantly at work in combining into new forms those elements which have been by death liberated; yet this fullness of life and its renovation differ according to climate*" (Humboldt and Bonpland 1807, page 215).

The gradual variation in temperature and rainfall along gradients of latitude and altitude remains the oldest recognised large-scale ecological pattern (Hawkins 2001), however most environmental factors experienced by organisms in nature follow the same pattern of gradual variation (and can therefore be referred to as 'environmental gradients'; Riesch et al. 2018), both in the case of abiotic factors (e.g., gradual variation of salinity or oxygen availability along stream gradients; Jacobsen et al. 2003, Piscart et al. 2005), biotic factors (e.g., gradual variation of food availability or predation risk; Esteves et al. 2008, Deacon et al. 2018), or even combinations of both (e.g., Kraft et al. 2011, Brown 2014, Egea-Serrano et al. 2014, Riesch et al. 2020).

Traditionally, most studies investigating the phenotypic effects of environmental variation have treated ecological factors as binary ("presence vs. absence"), focusing on the extremes of the considered variation, but *de facto* excluding everything in between (Riesch et al. 2018). Famous examples of such studies include phenotypic variation in "high- vs. low-predation" (e.g., Reznick and Endler 1982) or "toxic vs. non-toxic" environments (e.g., Riesch et al. 2014). These studies allowed us to establish general patterns showing how contrasting environments impact biodiversity; nevertheless, the reality appears to be much more complicated, as environmental variation has been shown to be gradual even in apparently simple systems (see for instance Riesch et al. 2016, Deacon et al. 2018).

The realization of the prevalence of environmental gradients in nature leads to several further research questions, which have been subject to considerable research effort only recently (Riesch et al. 2018). For example, even though environmental variation might occur in the form of a (more or less) linear gradient, does phenotypic divergence necessarily follow the same gradual pattern? This could apply whenever the optimal phenotype changes gradually, leading to continuous, small-scale local adaptations (Kawakami et al. 2011, Torres Dowdall et al. 2012, Riesch et al. 2016). Alternatively, phenotypic responses could be binary, where all changes happen whenever the environmental gradient reaches a particular threshold level, which would happen if the fitness of hybrid phenotypes is reduced (Hatfield and Schluter 1999, Via et al. 2000, Rundle 2002, Jacquemyn et al. 2018). In both cases, these processes do not only influence microevolutionary patterns, but also community structure, and potentially speciation (Nosil 2012).

Lastly, it is likely that multiple environmental gradients impact populations at the same time (Riesch et al. 2018). This can greatly influence our ability to detect which environmental factor(s) is/are actually driving observed phenotypic diversification, as the effects of multiple gradients can sum each other, or cancel each other out, or even a single, strong selective pressure can overshadow the effects of other environmental gradients. This must be considered, as it is possible for researchers to only focus on the most striking environmental difference within habitats, while other (ignored or not quantified) factors might actually be driving phenotypic diversity, or at the very least play an important role as well (Riesch et al. 2018).

Predictable responses to environmental gradients

While Humboldt described how environmental variation affects biological organisms, only in 1859, the year of his death, did the publication of Darwin's theory of evolution by natural selection (Darwin 1859) provide scientists with a predictive evolutionary framework to study the effects of environmental gradients. In the ca. 150 years since Darwin's publication, scientists have remained fascinated with being able to predict evolutionary outcomes, and/or which environmental conditions allow for predictable evolutionary differentiation (Langerhans 2010). Indeed, while several authors have argued in favour of the unpredictability of evolution (Gould 1989, 2002, Mani and Clarke 1990, Grant and Grant 2002, Pievani 2016) due to its inherently historical nature (see for instance Gould's thought experiment of "replaying the tape of life", Gould 1989), repeatable and predictable evolutionary outcomes have been highlighted for multiple taxa at different levels of biological organization (Conway Morris 1998, 2003, Losos et al. 1998, Schluter 2000, Hoekstra 2006).

Darwin himself predicted the existence of a "*yet unknown insect species*" – eventually identified as the sphinx moth *Xanthopan morganii praedicta* – with a proboscis long enough to pollinate the Madagascar orchid *Angraecum sesquipedale*, whose nectary is around 30 cm deep (Darwin 1862, Kritsky 1991). In recent years, studies on convergent evolution, experimental evolution and evolutionary genetics (Lobkovsky and Koonin 2012, Orgogozo 2015), have shown that evolutionary biology can be both experimental and predictive (Lässig et al. 2017), and ever-growing evidence revealed that evolutionary responses to environmental gradients are indeed predictable at many different levels, from molecules to populations (Stern and Orgogozo 2009, Riesch et al. 2014, Ujvari et al. 2015), at least under certain circumstances (Conway Morris 2010, Orgogozo 2015, Rainey et al. 2017).

Nevertheless, predicting organisms' responses to environmental variation can be a very difficult task, owing to the inherent complexity of both biological organisms and of the environments they live in (Langerhans and DeWitt 2004). Other than having a sufficiently good understanding of the selective pressures acting on an organism in each specific environment, and provided that evolutionary responses are not overshadowed by other, non-selective factors like genetic constraints or genetic drift (Losos et al. 2006, Blount et al. 2008, Langerhans and Riesch 2013), a good approach to be able to predict phenotypic responses to environmental gradients is to reduce the complexity of the phenotypes by focusing on the variation of a small suite of traits that are integral to adaptation to novel environments (Langerhans 2010).

This would allow us to derive sets of explicit *a priori* predictions on the variation of each given trait, which can be subsequently tested in field studies and/or laboratory experiments.

The analysis of life-history trait variation has been one of the most powerful tools for the prediction of phenotypic responses to environmental gradients (Reznick and Endler 1982, Stearns 1989, 2000). The theory is based on an optimization approach (Parker and Maynard Smith 1990), where organisms maximise their fitness in a particular environment by differentially allocating resources to a set of so-called life history traits, i.e., traits that directly impact individual fitness, e.g., growth rate, age and size at maturation, investment into reproduction, size and number of offspring and senescence (Stearns 1989). Since the overall amount of resources available to each individual is limited (but see Reznick et al. 2000), trade-offs are expected to emerge between different life-history traits (Stearns 1989). Environmental variation can affect these trade-offs by shifting the balance in favour of a particular trait. For example, increased mortality, as in the case of increased predation risk, is expected to favour traits that maximise early reproduction (e.g., younger age at sexual maturation, higher fecundity and reproductive investment) at the expense of other life-history traits (e.g., delayed sexual maturity, increased life span, Stearns 1989). Indeed, life-history theory has been able to accurately predict how organisms react to a wide range of environmental gradients, both in the field and in the laboratory (Roff 2002).

Why and how to predict the effects of environmental gradients?

Being able to answer the question “when habitats change, will organisms be able to adapt and, if so, how?” remains extremely important not only from a theoretical point of view, but also in light of the ever-increasing habitat changes that occur as a result of human activities (Magurran and Dornelas 2010, Pelletier and Coltman 2018). Humans are now widely regarded as one of the strongest evolutionary forces on the planet (Palumbi 2001, Bull and Maron 2016, Hendry et al. 2017), and are nowadays the main cause of environmental variation, which, as a consequence, is happening at an unprecedented pace (MacCracken 2009). Among the main sources of environmental variation are habitat fragmentation and destruction (Cheptou et al. 2017), pollution (Hamilton et al. 2017), global climate changes (Visser 2008, Lancaster et al. 2017), and the introduction of alien species into ecosystems (Lancaster et al. 2017). As habitats change, populations must respond to the new environmental conditions in order to avoid population collapse and ultimately extinction, and in fact organisms are often able to cope with environmental change by undergoing rapid phenotypic diversification. Phenotypic changes can occur either through phenotypic plasticity (i.e., the ability of a single genotype to produce different phenotypes depending on the environments; West-Eberhard 2003, Ghalambor et al. 2007, Lande 2015), or rapid evolutionary (i.e., genetic) change (Hendry et al. 2017, Reznick et al. 2019), or a combination of both, as phenotypic plasticity can influence rates of evolutionary change (Levis and Pfennig 2016, Fox et al. 2019), even leading to speciation (Bull and Maron 2016).

Studies that focus on phenotypic change along environmental gradients allow us to understand which environmental factors drive phenotypic differentiation among populations. Potentially, it would be possible to predict how organisms might react to global warming (Visser 2008, Hoffman and Sgrò 2011, Ożgo and Schilthuizen 2012), and life-history theory in particular has been proposed to be a useful tool to study the impact of climate changes (Fournier-Level et al. 2016, Lancaster et al. 2017). Similarly, more and more studies are investigating whether human-induced habitat fragmentation—another major threat to ecosystems—impacts the organisms' life-histories in a predictable way (e.g., Heinen-Kay et al. 2014, Riesch et al. 2014, Cheptou et al. 2017). Pollution not only has potentially devastating impacts on population survival but is also an important source of rapid phenotypic changes, often in a predictable fashion (Alexander et al. 2017, Hamilton et al. 2017). Lastly, the introduction and the success of invasive species causes well-known and widespread damage worldwide (Lowe et al. 2000). At the same time, however, rapid, predictable phenotypic evolution is one of the key factors for invasion success of alien species, as well as for the co-existence between native and invasive species (Colautti et al. 2017). Overall, these studies found a high degree of predictability of responses to anthropogenic sources of habitat change.

Being able to predict how organisms might change their phenotypes in order to respond to habitat changes has therefore important consequences for conservation biology (Colautti et al. 2017, Hendry et al. 2017, Lancaster et al. 2017), and allows us to better understand how these changes might impact ecosystems in the long term, or inform policy makers for planned interventions to better sustain biodiversity (Carroll et al. 2014, Smith et al. 2014).

Phenotypic effects of environmental gradients in invertebrates

Multiple studies have examined the influence of several environmental gradients on predictable phenotypic divergence in invertebrates. Among the main examples is the introduction of the fruit fly *Drosophila subobscura* to the Americas. The native range of these flies spans a large-scale latitudinal gradient, from North Africa to Scandinavia (Gilchrist et al. 2001). Throughout their range, flies increase in body and wing length from southern to northern populations, and these clines has been linked with increased survival of bigger individuals in colder climates (Huey et al. 2000, Gilchrist et al. 2001). Increased body size at higher latitudes was originally only predicted for endotherms by Bergmann's rule (Bergmann 1847), but has recently been found in many (but far from all, Mousseau 1997) ectothermic species (e.g., the ant *Leptothorax acervorum*, Heinze et al. 2003). Starting from the 1970s, *D. subobscura* was accidentally introduced to both South and North America, and spread throughout the continent once more along a replicated latitudinal gradient (Huey et al. 2000, Gilchrist et al. 2001). The invasion of *D. subobscura* represents a very important, unintentional natural experiment of evolution (Ayala et al. 1989, Stockwell et al. 2003, Colautti et al. 2017) that allows us to study patterns of shared and unique differentiation in independently evolving populations subject to the same environmental pressures (Langerhans and DeWitt 2004). In their invasive range, species are often also subject to novel environmental pressures (Suarez and Tsutsui 2008), and must undergo rapid phenotypic change in order for the colonization to be successful (Burton et al. 2010). Many invasions are also relatively recent, which provides us with opportunities to study evolution while it is happening (Burton et al. 2010). The analysis of body and wing length in the introduced populations of *D. subobscura* 20 years after introduction showed an increase in body size as a function of latitude as predicted (Huey et al. 2000, Gilchrist et al. 2001). However, New-World and Old-World *Drosophila* achieve bigger wing length by increasing two different and unrelated parts of the wing (Huey et al. 2000). Subsequent studies on *D. subobscura* showed that populations collected from the extremes of the species native range (Netherlands and Spain), converged predictably towards a mean body size when housed at a constant intermediate temperature in the laboratory (Simöes et al. 2017), which suggests the presence of strong, divergent selection across their range. Similar clines of body size have also been found in *D. melanogaster* (where they have been observed across all continents, Gilchrist et al. 2001), as well as other species of *Drosophila* (Huey et al. 2000).

These large-scale biogeographical patterns are however not ubiquitous. For example, in native populations of the seven-spotted lady beetle (*Coccinella septempunctata*) the size of black spots (melanism) follows a latitudinal cline, with individuals from northern populations being more pigmented than those from southern populations (O’Neill et al. 2017). Similar clines are not, however, present in populations sampled from its invasive American range (O’Neill et al. 2017), highlighting how predictable evolution across geographical gradients might depend on the strength of selection acting on each particular trait.

Long-term natural experiments can also be used to study phenotypic responses to climate. An example of this is represented by the shell colouration of the snail *Cepea nemoralis*, which strongly affects thermoregulation (Ożgo and Schilthuizen 2012). Lighter shells are advantageous in hot and exposed conditions, whereas darker shells are favoured in dark and shady habitats, since they offer better camouflage against bird predators (Ożgo and Schilthuisen 2012). In an introduced population of *C. nemoralis* from Poland, shell colouration showed a strong correlation with habitat type, providing a strong evidence for contemporary adaptation of shell colour to the local environment (Ożgo and Kinninson 2008). In a different study, the same authors analysed shell colouration in *C. nemoralis* collected from multiple sites in the Netherlands across a period spanning 43 years, and compared it with temperature changes over the same period. Climate data showed an increase in mean temperature across the 43 years, and predictably snails evolved increasingly lighter shells in response to the higher temperatures, independently from habitat composition (Ożgo and Schilthuisen 2012).

Phenotypic effects of environmental gradients in vertebrates

Vertebrates have been the subject of countless studies investigating the effects of environmental gradients. On a large geographical scale, ecologists have recognised multiple so-called ecogeographical rules in order to explain several aspects of phenotypic variation, especially along latitudinal gradients (Gaston et al. 2008). One of the main ecogeographical rules is Bergmann’s rule, which describes the increase in body size observed in multiple species of endotherms at higher latitudes (Bergmann 1847). The pattern has been related to advantages in thermoregulation experienced by bigger individuals in colder climates, due to the more favourable body surface-to-volume ratio (Meiri and Dayan 2003). Bergmann’s rule has been found to be true for a wide range of mammals and birds. For instance, Swedish moose (*Alces alces*) populations increase in average body size from southern to northern populations (Sand et al. 1995).

In addition to being a general pattern across space, Bergmann’s rule has been reported in populations across historical and evolutionary time when exposed to different thermal regimes (Smith et al. 2000). Present-day American woodrats (*Neotoma* spp.) conform to Bergmann’s rule, but the relationship between temperature and body size has been found to persist even in the fossil record, as during the last 20,000 years woodrats were relatively smaller during warmer periods, and bigger during glacial maxima (Smith et al. 2000). The temporal relationship between body size and temperature can also be exploited for the study of phenotypic responses to contemporary global climate changes. Recently, Weeks et al. (2019) demonstrated that 52 species of North American migratory birds significantly reduced their body size over the last 40 years as a consequence of global warming.

Large-scale phenotypic responses to latitudinal temperature gradients can also involve allometric body shape responses, as in the case of Allen’s rule. It posits that in endotherms, individuals living at higher latitudes will have relatively shorter limbs for their body size than those living at lower latitudes (Allen 1877). While support for Allen's rule is less widespread than for Bergmann's rule (Nudds and Oswald 2007), it has been found to hold true for multiple populations of 43 species of seabird (Nudds and Oswald 2007), as well as for multiple human populations (Katzmarzyk and Leonard 1998).

Gloger's rule (Gloger 1833), on the other hand, states that, within endotherm species, more pigmentated individuals should reside in more humid environments, in particular towards the equator. Increased pigmentation in humid habitats appears to be favourable for birds and mammals, as darker feathers are more resistant to bacterial infections and provide increased photoprotection (Burtt and Ichida 2004). Indeed, over 96% of North American bird species follow this rule (Zink and Remsen 1986), and the same pattern has been confirmed also for several species of mammals such as primates (Kamilar and Bradley 2011), artiodactyls (Stoner et al. 2003), carnivores (Ortolani and Caro 1996) and house mice (*Mus musculus*, Lai et al. 2008).

Environmental unpredictability, in particular along a rainfall gradient, has been shown to drive social evolution in African mole rats (Bathyergidae), a family of subterranean rodents native of sub-Saharan Africa (Bennett and Faulkes 2000). Among African mole rat species exhibit multiple types of social structure: some species are solitary, others are social, and two (the naked mole rat, *Heterocephalus glaber*, and the Damaraland mole rat, *Fukomys damarensis*) are eusocial (Bennett and Faulkes 2000). Mole rat social evolution is thought to be driven by habitat aridity and food distribution

(Faulkes and Bennett 1997, 2007, Bennett and Faulkes 2000) along a rainfall gradient. In arid environments, group living is selected for, as the lack of rainfall leads to harder soil—which increases the costs of digging as well as patchy distribution of food resources, and thus, increases the risk of unsuccessful foraging (Bennett and Faulkes 2000). Indeed, solitary species are found in mesic environments, whereas social and in particular eusocial species inhabit dryer environments (Faulkes and Bennett 1997, 2007, Bennett and Faulkes 2000).

Among reptiles, Caribbean *Anolis* lizards are an important model for the study of convergent evolution (Langerhans et al. 2006). In each of the four Greater Antilles islands they have independently diverged into different species that have colonised similar habitats, with species living in the same habitat in different islands being characterised by similar morphology, ecology, and behaviour (Langerhans et al. 2006).

One of the *Anolis* species, *A. sagrei*, is often found on the ground in the absence of predators, but has been known to transition to a more arboreal lifestyle if predators, in particular *Leiocephalus carinatus*, are present (Schoener et al. 2002). In 2003, Losos and colleagues introduced *L. carinatus* to six, previously predator-free islands near Great Abaco, The Bahamas (randomly choosing another six islands to serve as control), in order to study the predictability of these responses (Losos et al. 2006). They measured *A. sagrei* survival and morphology twice, six months apart. They predicted that *A. sagrei* would first develop longer limbs to better escape the predator, and only then adopt a more arboreal lifestyle, leading to a reversal of selection and to the evolution of shorter limbs, more useful for locomotion in trees

(Losos et al. 2006). Across all six islands, these predictions were confirmed, as the authors found that the relative limb length of *A. sagrei* increased six months after the predator introduction, and subsequently decreased six months later, while it remained constant in the six control islands (Losos et al. 2006).

Fish are an extremely important vertebrate group for the study of predictable phenotypic responses to environmental gradients. Many freshwater fish are subject to so-called stream gradients (Langerhans 2008, Lostrom et al. 2015). From upstream to downstream, fish experience gradual variation in several biotic and abiotic environmental factors, such as predation risk (Deacon et al. 2018), temperature (Jourdan et al. 2016), and water speed regime, which decreases along the river course (Langerhans 2008). For example, Langerhans (2008) analysed the influence of flow regime on fish morphology using a meta-analysis of 83 different studies. Theory predicts that fishes from environments characterised by high flow regimes should exhibit increased steady swimming performance compared to those living in slow-moving streams, in order to resist strong currents. Overall, these responses were found to be highly predictable and shared not only across different taxa, but also across multiple morphological traits (Langerhans 2008).

Moreover, Hendry (2001) studied adaptive variation in body size in recently introduced populations of sockeye salmon (*Oncorhynchus nerka*) in relation to sexual section and flow speed. In this species, sexual selection favours males with big and deep bodies, however, deep bodied males are at a disadvantage in shallow rivers and fast flowing waters. In females, larger body size is selected for because larger females produce more eggs and are favoured in the competition to obtain high quality nesting sites. At the same time, however, bigger females are more likely to get stranded and die in shallow fast-flowing rivers, where they are instead selected for smaller body sizes. The analysis of body size and shape of salmons from two recently-established populations from Canada confirmed the presence of divergent selection that led to predictable phenotypic differentiation, as salmon from shallow, fast-flowing rivers were consistently smaller and had less-deep bodies than those from the slow-flowing river (Hendry 2001).

Phenotypic effects of environmental gradients in livebearing fishes (Poeciliidae)

Livebearing fishes (Poeciliidae) are a family of more than 260 species of freshwater fish native to the Americas (Stockwell and Henkanaththegedara 2011). Among fish, poeciliids have been possibly the most-studied family across several different scientific fields, including studies focusing on evolutionary biology, ecology, behaviour, sexual selection, and more (Meffe and Snelson 1989, Houde 1997, Magurran 2005, Evans et al. 2011). The success of poeciliids as model organisms for both field and laboratory stems from their ability to cope with a wide variety of environmental conditions (e.g., differences in temperature, salinity, predation, or the presence of toxins in the water, Johnson and Bagley 2011), in their natural habitats, as well as being extremely robust and easy to breed in captivity (Evans et al. 2011). Several poeciliid species are also important as invasive species, and can be found almost worldwide, as a result of both accidental and intentional human introductions (Meffe and Snelson 1989, Pyke 2005, 2008, Deacon et al. 2011).

Within the context of predictable responses to environmental gradients, studies on shared versus unique, and predictable versus unpredictable, response to environmental variation (often with a focus on life-history traits) are most prominent (Reznick and Endler 1982, Reznick et al. 1990, Johnson and Belk 2001, Jennions et al. 2006, Marsh-Matthews and Deaton 2006, Zúñiga-Vega et al. 2007, Tobler and Hastings 2011, Torres-Dowdall et al. 2013, Jourdan et al. 2016, Riesch et al. 2018). Throughout the family, there is an extreme variation of life-history strategies, both among and within species (Johnson and Bagley 2011, Pires et al. 2011), even though all species share remarkably similar reproductive adaptations. In all male poeciliids, the anal fin is modified into a copulatory organ, the gonopodium, used to transfer sperm to females for internal fertilization, while females (with the exception of the oviparous *Tomerus gracilis*; Johnson and Bagley 2011) are viviparous and give birth to live and independent offspring (Evans et al. 2011). Over the years, multiple studies have used the association between life-history traits and ecological conditions to show that life-history differences are the result of natural selection (e.g., Reznick et al. 1990, Riesch et al. 2010, Johnson and Bagley 2011).

Typically, studies focusing on poeciliid life histories have concentrated on variation in a relatively small set of life-history traits, such as male and female body size, reproductive investment (in both sexes: testis weight relative to body weight in males, and offspring weight relative to body weight in females), fecundity, offspring size, but also presence or absence of superfetation (i.e., the ability of carrying several broods simultaneously at different developmental stages), or the degree of maternal provisioning to the offspring following fertilisation, which varies from lecithotrophy, where all nutrients are stored in the eggs before fertilization, to matrotrophy, where nutrients are provided by the mother to the offspring during development (Reznick and Endler 1982, Reznick et al. 1996, Johnson and Bagley 2011, Riesch et al. 2013). These life-history traits accurately describe reproductive strategies across species or populations and are expected to be subject to trade-offs dependent on different ecological conditions, as predicted by life-history theory (Roff 1992, Stearns 1999, 2000). Indeed, the same environmental gradients have oftentimes caused parallel life-history differentiation across multiple species or multiple populations of the same species (Johnson and Bagley 2011).

One of the most researched life-history trade-offs is the one between fecundity and offspring size, which arises partially as a consequence of viviparity, as female poeciliids have limited body cavity space available for the developing embryos (Smith and Fretwell 1974, Qualls and Shine 1995), thus selection for increased embryo size is usually accompanied by reduced fecundity, and vice versa. When adult mortality is high relative to juvenile mortality (e.g., in the case of high predation risk), theory predicts that females maximize their fitness by increasing fecundity which should also result in a corresponding reduction in offspring size (Stearns 1992, Roff 1993). On the other hand, if bigger offspring are selected for (e.g., in the case of increased competition for resources in low-quality environments; Rollinson and Hutchings 2013), fecundity is expected to decrease (Stearns 1989, Riesch et al. 2014). Multiple environmental conditions can affect this trade-off, as reviewed by Moore et al. (2016). Using the meta-analysis approach, the authors evaluated the predictability of offspring size and fecundity variation in response to differences in predation risk, population density, food availability, salinity and water toxicity due to hydrogen sulphide across 54 studies on 17 species. However, they found predictable life-history divergence only in response to variation in predation regime and concentrations of hydrogen sulphide in the water, while responses to population density, food limitation and salinity were either less predictable or at the very least not significantly different from zero (Moore et al. 2016).

Life-history responses to variation in predation risk have been studied extensively for several decades and have been highlighted in several species of livebearing fish (most prominently the guppy, *Poecilia reticulata*, Reznick and Endler 1982; but also in *Brachyraphis rabdophora*, Johnson and Belk 2001; or Bahamas mosquitofish, *Gambusia hubbsi*, Riesch et al. 2012, 2020), and we know more about the effects of predation on life-history evolution in poeciliids than about any other environmental variable (Johnson and Bagley 2011). In high-predation sites, fish are subject to high levels of extrinsic mortality (Reznick and Endler 1982) and are predicted to be characterised by *r*-selected life-history phenotypes (Pianka 1970, Reznick et al. 2002) with small body size and low body fat coupled with high reproductive investment, high fecundity and small offspring size compared to individuals from low-predation sites. In Trinidad, guppies from high-predation environments co-occur with large piscivorous fish, such as the pike cichlid *Chrenicicla alta*. Low-predation sites occur in the same drainages, upstream of waterfalls that exclude large predators. Here, guppies co-occur instead with the killifish *Anablepsoides hartii* (previously known as *Rivulus hartii*), which tends to feed on juveniles (Reznick et al. 2002). Analysing guppy life-history traits in nine low-predation and seven high-predation sites from northern Trinidad, Reznick and Endler (1982) showed that guppy phenotypes ideed changed in response to the different predation pressure in the predicted direction. Furthermore, in 1976, John Endler had transplanted guppies from a high-predation population to a stream that previously only housed *A. hartii* (and was therefore a low-predation site). David Reznick and colleagues repeatedly sampled these guppies between 1981 and 1988, and found that, in the span of a few generations, they had shifted their life-history strategy in the predicted direction, and were now characterised by a "low-predation phenotype", with females in the introduced population having a reduced reproductive investment and giving birth to fewer, bigger offspring than females from the original population (Reznick et al. 1990). Also, by rearing guppies from both populations in the laboratory under common-garden conditions, they were able to demonstrate that these life-history differences were the result of (rapid) genetic evolution, and not just of phenotypic plasticity (Reznick et al. 1990). Subsequent studies showed that the same evolutionary responses occurred independently in several other rivers throughout the island of Trinidad (Reznick et al. 1996).

While traditionally these studies have identified high and low predation as a binary condition (i.e., presence vs. absence of predators), it has recently become apparent that in reality this is more of a continuous gradient of predation risk (Deacon et al. 2018). Nevertheless, guppies still show small-scale life-history adaptation to the gradient of predation risk in six localities along a river in Trinidad (Torres Dowdall et al. 2012). Moreover, several other environmental gradients have been identified as having an influence on phenotypic responses in "high- and low-predation guppies", namely canopy cover, food availability, and population density (Endler 1995, Grether et al. 2001). Mathematical models have in fact suggested that, if the predation risk gradient were the only selective force acting on these populations, "high-predation phenotypes" should outcompete "low-predation" ones even when the predation risk is low (Grether et al. 2001). However, low-predation sites are also characterised by high population densities and reduced food availability (which is in turn a consequence of increased canopy cover). Thus low-predation phenotypes are not only the consequence of predation pressure, but also of density-dependent selection driven by food availability (Grether et al. 2001).

Similarly, Jourdan et al. (2016) examined phenotypic differentiation in two closely related species of *Gambusia* from Mexico (*G. sexradiata* and *G. yucatana*), along a stream gradient, consisting of both a predation and a climate gradient. While predation differences drove consistent phenotypic patterns in both species, other components of the gradient influenced each species in a unique, unpredictable way. This highlights how selective pressures in these environments are complex, and reinforces that multiple environmental gradients are likely to influence phenotypic differentiation simultaneously (Jourdan et al. 2016).

Other environmental gradients have also been studied with regard to their predictable effects on poeciliid life-history. Among those are hydrogen sulphide-induced water toxicity, salinity or food availability (Tobler and Plath 2011). Hydrogen sulphide (H_2S) is an extremely powerful toxicant that interferes with mitochondrial respiration and blood oxygen transport, and also causes extreme hypoxia in the water (Bagarinao 1992, Grieshaber and Völkel 1998). It can be derived from volcanic activity, bacterial decomposition in anoxic environments or human activities. Poeciliids have repeatedly colonized toxic, hydrogen-sulphide rich waters across their natural distribution (Riesch et al. 2016). Life-history adaptations to H_2S have been studied in multiple *Gambusia* and *Poecilia* species (Riesch et al. 2014, 2016) that have colonized sulphide-rich springs independently in the United States, Caribbean, Mexico, and Venezuela. In low-quality environments, life-history theory predicts that fish should produce bigger offspring (Rollinson and Hutchings 2013). Moreover, bigger offspring should be further selected for in toxic environments due to their more favourable volume-to-surface area ratio (Powell 1989). In both cases, an increase in offspring size is expected to be accompanied by a reduction in fecundity. These predictions have been confirmed in most (but not all) cases of independent colonization of H_2S-toxic springs, throughout the entire poeciliid range (Riesch et al. 2014, 2016). Nonetheless, the extent of these responses has been shown to vary greatly in response to the gradient of H_2S concentration found in each population (Riesch et al. 2016), as well as being achieved through different, not always predictable, molecular adaptations (Pfenninger et al. 2014, 2015).

Salinity is another environmental factor that can form extensive gradients and causes several physiological problems to most fish (Birnie-Gauvin et al. 2017), especially osmotic stress (Moore et al. 2016).

Nevertheless, poeciliids can tolerate a wide range of salinities (Chervinski 1984, Nordlie et al. 1992), from freshwater to salinity levels even higher than ocean water (Tobler and Plath 2011). While salinity variation is predicted to select for larger adult body size and offspring size, both of which would be advantageous in coping to osmotic stress due to a more favourable body volume-to-surface ratio (Moore et al. 2016), phenotypic responses to salinity gradients in poeciliids are overall not consistent and predictable (Moore et al. 2016). Nevertheless, in invasive eastern mosquitofish, *Gambusia holbrooki*, females from high salinity habitats were characterized by higher reproductive investment and produced larger offspring when compared to females from freshwater habitats (Alcaraz and García-Berthou 2007). Also, in *G. hubbsi*, males living in high salinity environments had higher lean weight than males in low salinity ones (Riesch et al. 2015). The lack of consistent responses across species, contrary to what was observed for predation or H_2S gradients (Moore et al. 2016), could be explained by the fact that the strength of selection due to salinity might be lower than that from the other two gradients (e.g., Reznick et al. 1996, Plath et al. 2013). For example, life-history responses along a salinity gradient in Brazilian *Poecilia vivipara* appear to be actually mediated by differences in predation pressure between high- and low-salinity environments, due to the fact that the main predator species in the system are extremely salinity intolerant and are therefore not present in the high-salinity populations (Gomes-Jr and Monteiro 2007).

Life-histories are not the only suite of phenotypic traits that lend themselves to studies on predictable responses to environmental gradients. Similar to other fish families, multiple studies have investigated how environmental gradients influence body shape differentiation in livebearing fishes (Langerhans 2008, Langerhans and Reznick 2009), in particular using geometric morphometrics (Rohlf and Marcus 1993, Adams et al. 2004). In all fishes, body shape is a strong determinant of individual swimming performances (Langerhans and Reznick 2010). More streamlined body shapes, characterised by a deep/wide anterior body and shallow/narrow caudal peduncle gives the fish higher endurance and higher performances in steady-swimming, whereas bigger caudal peduncles and smaller heads provide better performances in acceleration and escape bursts (fast-start swimming; Langerhans 2008). In low predation conditions, natural selection is expected to favour steady swimming, thus increasing competitive ability and food acquisition. In contrast, fast-start swimming should be selected for in high predation environments, hence increasing flight-response and survival probability in encounters with predators (Langerhans 2008). The predictability of body shape responses to predation gradients has been tested by Langerhans (2010) in *Gambusia affinis* and *G. hubbsi* (see also Langerhans et al. 2004, 2007). Fish were always characterised by larger and deeper caudal peduncles, and relatively smaller heads, when subject to high predation risk, while the opposite was true in low-predation environments (Langerhans 2010). The same body shape responses to predation have also been found in several other poeciliid species (*Gambusia caymanensis*, Langerhans and Makowicz 2009; *B. rabdophora*, *P. reticulata*, Langerhans and DeWitt 2004; *Poecilia mexicana*, Tobler et al. 2008; *P. vivipara*, Gomes and Monteiro 2008), but also in non-poecilids like the threespine stickleback *Gasterosteus aculeatus* (Walker and Bell 2000). Interestingly, while the overall phenotypes were the same, different species achieved it through different mechanisms: larger caudal regions were the consequence of elongation of such region in certain species (e.g., in *G. holbrooki*, Langerhans et al. 2004), but in other species they were achieved through deepening (e.g., *G. hubbsi*, Langerhans et al. 2007), or a combination of the two (Langerhans 2010).

As in the case of life-history evolution, poeciliid body shape often responds in a predictable way not only to predation, but also to several other environmental gradients, for instance, H_2S-induced water toxicity. Fish have evolved multiple behavioural and morphological adaptations in response to the highly hypoxic environment caused by the hydrogen sulphide (Plath et al. 2007, Tobler et al. 2009, Riesch et al. 2016). Several species inhabiting toxic sulphur springs rely on aquatic surface respiration in order to extract oxygen from the interface between water and air, where it is most abundant (e.g., *P. mexicana*, Tobler et al. 2011). At the same time, on a morphological level, these individuals are characterised by increased head size (Tobler and Hastings 2011), which in turn allows for a larger gill region, thus increasing oxygen acquisition efficiency during aquatic surface respiration (Plath et al. 2007, Tobler et al. 2009). Such increases in gill size have also been shown in several other aquatic organisms inhabiting toxic environments (Langerhans et al. 2007, Tobler et al. 2011). While this pattern has been found to be widespread and largely predictable in multiple *Poecilia* and *Gambusia* species (Riesch et al. 2016), different populations were characterised by additional population-specific shape differences (Riesch et al. 2016). In particular, the amount of population differentiation in toxic sites depended on the concentration of hydrogen sulphide in each population, similarly to life-history responses to the same environmental gradient (Riesch et al. 2016).

Importance of livebearing fishes as invasive species

One final, important aspect of poeciliid ecology is their relevance as invasive species. Several species, in particular guppies and eastern and western mosquitofish are among the most dangerous invasive alien species (IAS) worldwide (Lowe et al. 2000). They have been repeatedly introduced both accidentally, as a result of the pet trade (Deacon et al. 2011), and intentionally, as mosquito-control agents (Pyke 2005, 2008). Consequently, they can now be found in every continent except Antarctica (Stockwell and Henkanaththegedara 2011). Once established, poeciliids can reach high population densities and are extremely difficult to eradicate, while at the same time causing well-established, negative effects on local fauna (Kottelat and Whitten 1989).

Nonetheless, they also provide invaluable, large scale natural experiments of contemporary evolution, as they must be able to respond to any novel environmental conditions they encounter during the invasion of a new habitat (e.g., Alcaraz and García-Berthou 2007, Benejam et al. 2009, Ouyang et al. 2018). Moreover, it is possible to compare patterns of phenotypic diversity between their invasive and their native ranges. This can provide important information both on which traits might be important to invasion success (Cote et al. 2010), as well as on the evolutionary processes involved in rapid diversification due to environmental gradients.

From a behavioural point of view, for example, invasive *G. holbrooki* and *G. affinis* had significantly higher boldness and dispersive tendency than the non-invasive *Gambusia geiseri* and *Gambusia hispaniolae*, despite strong similarities in morphology, ecology and body size (Rehage and Sih 2004). From a morphological and life-history standpoint, phenotypic variation along a north-south latitudinal gradient has been investigated in *G. holbrooki* from their native American range (Riesch et al. 2018), as well as in invasive *G. affinis* from China (Ouyang et al. 2018). Along their native range, phenotypic diversification in *G. holbrooki* was mainly driven by climatic gradients, with individuals from northern populations being characterised by increased body size, larger reproductive investment, smaller offspring, and shallower and more streamlined bodies than those from southern populations (Riesch et al. 2018). However, life-history and body-shape variation was also partly driven by climate-independent environmental factors such as population density and habitat productivity (Riesch et al. 2018). Similar phenotypic changes in response to a North-South climatic gradient were also found in invasive *G. affinis* from China (Ouyang et al. 2018). In those individuals, however, an inland-coastal environmental gradient also contributed to phenotypic diversity (Ouyang et al. 2018).

Conclusions, open questions and future directions

Being able to accurately predict how organisms react to environmental change, while having been the subject of increased research efforts over the last few years (Lässig et al. 2017), remains one of the main aims of evolutionary biology. All the examples discussed so far in this review prove that, at least in some cases, it is possible to predict the results of organismal responses to environmental gradients (Lässig et al. 2017). Widespread, convergent responses across populations and/or taxa subject to similar selective pressures are being described more and more often (Conway Morris 2010, Riesch et al. 2016, Rosenblum et al. 2017).

Nevertheless, several questions remain unanswered (Conway Morris 2010, Lässig et al. 2017), and many studies continue to highlight the importance of historical contingency in evolution (Tyerman et al. 2005). For instance, how do multiple environmental variables interact in driving phenotypic differentiation among populations? What drives "unique features of evolutionary differentiation" experienced by populations subject to the (seemingly) same environmental gradients (Langerhans and DeWitt 2004), and how consistent are phenotypic responses to environmental gradients across different study systems? Lastly, is it possible to predict how populations will respond to human-induced habitat modifications?

One possible approach to answering these questions is to concentrate on multiple, independently evolving populations (or species) subject to similar environmental pressures, in order to understand the nature of the selective pressures acting in particular habitats. Furthermore, since populations are usually subject to multiple, interacting environmental factors (Langerhans and Makowicz 2009) that differentially affect several phenotypic traits, a multi-trait, multi-factor approach has recently been proposed as the best tool to study predictable evolution (Langerhans 2018). This approach would allow us to evaluate the relative importance of multiple environmental gradients, as well as the contribution of historical contingency in shaping organisms' diversity (Langerhans and DeWitt 2004). This framework has since been applied several times with good results (e.g., Gilchrist et al. 2001, Langerhans et al. 2006, Langerhans and Makowicz 2009, Riesch et al. 2016, 2020, Langerhans 2018).

Poeciliids, thanks to their diversity and wide distribution across multiple environmental gradients, provide the unique opportunity to investigate phenotypic responses to similar gradients across multiple species, to compare responses to environmental gradients on different spatial scales, and to compare the predictability of responses to arguably strong versus relatively weak environmental selective forces. Therefore, a wide range of questions regarding how, when and why evolution may be predictable can be answered thanks to the different aspects of poeciliid biology.

Overview of research chapters

In the following research chapters I aimed to investigate the knowledge gaps highlighted above. Specifically, in **Chapter 2**, I analysed how multiple environmental gradients interact even at a relatively small geographical scale in driving phenotypic differentiation in *Phalloptychus januarius* populations from coastal lagoons in Brazil, focusing on the relative importance of each selective pressure to this species. In **Chapter 3**, I analysed the effects of climate-dependent and -independent environmental variation on phenotypic differentiation in invasive eastern mosquitofish, *G. holbrooki*. I firstly analysed morphological and life-history variation of wild-caught individuals from a large geographic area in the invasive range, spanning France, Italy and Spain. Secondly, using population genetics tools, I investigated the invasion history and range expansion of mosquitofish across Europe, and thirdly, I made use of data from a common-garden experiment to tease apart the different contributions of phenotypic plasticity and rapid evolutionary differentiation to phenotypic diversity. In **Chapter 4**, I analysed the effects of large-scale latitudinal gradients on a different phenotypic trait of invasive mosquitofish, namely the level of multiple paternity. Here, I used wild-caught eastern mosquitofish (*G. holbrooki*) from across Europe, and western mosquitofish (*G. affinis*) from China, and I investigated the relative contribution of climate to both geographical and temporal variation of multiple paternity. In **Chapter 5**, I investigated whether the presence of a single, strong selective pressure over an intermediate geographical scale increased the predictability of phenotypic differentiation. To that end, I explored morphological and life-history differences in guppies (*P. reticulata*) living in toxic, oil-polluted environments across Southern Trinidad. Finally, I synthetized and critically evaluated my results in a general discussion (**Chapter 6**), with special focus on new and remaining research questions, as well as future directions.

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Chapter 2: Phenotypic differentiation in a heterogeneous environment: morphological and life-history responses to ecological gradients in a livebearing fish

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Abstract

Predicting how environmental variation drives phenotypic diversification is one of the main aims of evolutionary ecology. Yet, we still only have a limited understanding of how it drives diversity, especially when multiple factors interact. To address this issue, the superfetating livebearing fish *Phalloptychus januarius* (Poeciliidae) was repeatedly sampled (over a two-year period) in four coastal lagoons in Brazil to investigate the relative contribution of different environmental factors on phenotypic patterns. We further compare our results to those reported for another poeciliid (*Poecilia vivipara*), which inhabits some of the same lagoons but has a drastically different reproductive strategy (no superfetation). We used a model averaging approach to estimate the relative importance of differences in predation pressure, salinity, dissolved oxygen, pH, temperature and food availability in driving variation in body shape, life histories, and life-history proxies. We found consistent population differences in several traits, but also seasonal variation within each lagoon. Specifically, predation, oxygen availability, and pH affected several different traits, and played important roles in driving phenotypic differences between and among populations. Moreover, our study reveals differential responses in phenotypic traits to the same environmental gradients between *P. januarius* (this study) and *P. vivipara* (previous studies), and we suggest that future work should further investigate differential phenotypic responses to single vs. multiple concomitant selective forces, and how this affects different species.

Key-words

Body shape, Environmental gradients, *Phalloptychus januarius*, Poeciliidae, Predictable evolution.

Introduction

Predicting how organisms respond to changes in their environment is one of the key aims of evolutionary biology (Stearns 1977, Langerhans 2018) but faces challenges when multiple (interacting) environmental parameters affect trait divergence (MacColl 2011, Langerhans 2018, Riesch et al. 2018). Nevertheless, we will be able to properly describe the whole extent of phenotypic responses to environmental variation only by simultaneously considering multiple environmental factors and traits (DeWitt and Langerhans 2003, Heinen et al. 2013, Langerhans 2018).

Traditionally, the study of predictable phenotypic evolution has focused on the effect of single environmental factors, often treating environmental variation as binary, such as high vs. low predation (Losos et al. 2006, Heinen et al. 2013), high vs. low salinity (Palkovacs et al. 2008), and other habitat characteristics (Kaeuffer et al. 2012). A particularly well-researched system in this regard is the Trinidadian guppy (*Poecilia reticulata*), and the effects that differences in predation pressure have on life histories (Reznick and Endler 1982, Reznick et al. 1990), morphology (Alexander et al. 2006), body colouration (Endler 1983) and behaviour (Seghers 1974). More recently, however, it has become apparent that not only do multiple environmental parameters often act in unison to shape phenotypic divergence, but also that environmental variation is in most cases not binary. Instead, environmental variation occurs as a (environmental) gradient, i.e., gradual change of environmental factors on both a spatial and a temporal scale (Riesch et al. 2018). For instance, in addition to predation, also food availability (Grether et al. 2001, Hendry et al. 2006) and competition (Bassar et al. 2016) affect guppy life histories. Moreover, predation pressure itself also varies gradually between the extremes represented by the traditional high- and low-predation environments (Deacon et al. 2018). This has important consequences in our ability to correctly predict how organismal responses to environmental variation as, for example, more than 50% of the total life-history variation of Bahamas mosquitofish (*Gambusia hubbsi*) subject to different predation regimes cannot be described by variation in predation pressure alone (Langerhans 2018).

Here, we investigated phenotypic differentiation of the livebearing fish *Phalloptychus januarius* (Hensel 1868) from coastal lagoons in southeastern Brazil with starkly different ecological conditions (Araújo et al. 2014) and diverse fish communities (Di Dario et al. 2013). Previous studies in this system focused on life-history and morphological variation in another poeciliid (*Poecilia vivipara*), in relation to gradients of salinity and predation (Neves and Monteiro 2003, Gomes-Jr and Monteiro 2007, Gomes and Monteiro 2008, Araújo et al. 2014, Rius et al. 2019). The main piscine predator of poeciliids, *Hoplias malabaricus*, cannot tolerate high-salinity conditions (Gomes-Jr and Monteiro 2007), while other predators, such as fish-eating bats *Noctilio leporinus* (Luz et al. 2011), birds (e.g., White-backed stilt, *Himantopus melanurus*, Great kiskadee, *Pitangus sulphuratus*, Black-crowned night heron, *Nycticorax nycticorax,* or various kingfishers) and insects (e.g., Belostomatidae) are present, yet not abundant (A. Petry, M. Plath and R. Riesch, personal observations). Predation pressure for all *P. januarius* age classes, therefore, is bound to increase from highly saline to freshwater lagoons (Gomes-Jr and Monteiro 2007). Still, other environmental factors—including oxygen availability, habitat productivity, and pH—also vary between lagoons, and could account for a substantial amount of the observed variation (Langerhans and DeWitt 2004).

We tested for phenotypic differences between fish from different lagoons as well as temporal differences within lagoons, and evaluated the relative importance of multiple environmental factors in driving these patterns. We focused on a species that has not been studied so far in this system, and compared our results to previous studies on *P. vivipara* from some of the same lagoons (sampled at the same time as the *P. januarius* our study focuses on) to identify shared and unique patterns of divergence to the same environmental gradients (Araújo et al. 2014, Rius et al. 2019). This is of particular interest as both species have radically different reproductive strategies: *P. vivipara* develop a single clutch per reproductive bout and are lecithotrophic (i.e., resources required for embryo development are stored in the eggs prior to fertilization; Arcanjo et al. 2014), while *P. januarius* are characterised by superfetation (i.e., females simultaneously bear two or more broods at different developmental stages) and matrotrophy (i.e., substantial maternal provisioning after fertilization; Pollux and Reznick 2011, Olivera-Tlahuel et al. 2015). Superfetation allows females to reduce body cavity space devoted to offspring production without reducing overall fecundity (Qualls and Shine 1995, Zúñiga-Vega et al. 2007). This could result in different evolutionary trajectories when both species are exposed to the same environmental gradient(s).

Correctly predicting how multiple environmental gradients interact to drive phenotypic differentiation is difficult due to their inherent complexity.

Thus, we formulated the following set of *a priori* predictions, based on what would be expected if each environmental factor was acting alone. Our statistical analyses test if these effects were still uncovered when multiple environmental factors vary simultaneously.

Prediction 1 (predation-effect): In low-predation lagoons, fish should be larger, have more body fat and larger offspring size at birth, but lower lean weight, fecundity, and reproductive investment compared to high-predation (low-salinity) sites (Stearns 1989, Reznick et al. 1990, Reznick et al. 2002b, Alexander et al. 2006, Riesch et al. 2015). Individuals from high-predation lagoons should have smaller heads but larger caudal peduncles compared to those from low-predation habitats (e.g., Langerhans 2009, Langerhans and Makowicz 2009).

Prediction 2 (salinity-effect): High salinity causes osmotic stress and should select for decreased body surface-to-volume ratios, i.e., increased body and offspring size (Alcaraz and García-Berthou 2007, Gomes-Jr and Monteiro 2007, Riesch et al. 2015, Moore et al. 2016). We further predicted higher salinity to be associated with higher fat content, increased fecundity and reproductive investment (e.g., *Gambusia holbrooki*, Alcaraz and García-Berthou 2007; *Poecilia latipinna*, *Gambusia affinis* and *Heterandria formosa*, Martin et al. 2009; but see Moore et al. 2016).

Prediction 3 (temperature-effect): Water temperature varies only to a minor extent between lagoons, but greatly between seasons, with warmer temperatures during the wet than during the dry season. We predicted that higher water temperatures will be associated with increased adult body size, fecundity and reproductive investment (Vondracek et al. 1988, Abney and Rakocinski 2004).

Prediction 4 (oxygen-effect): We predict low-oxygen conditions to be associated with larger head and gill regions to facilitate oxygen acquisition (Chapman 2015), and potentially also with reduced fecundity (Riesch et al. 2015).

Prediction 5 (food availability-effect): In habitats with greater productivity (estimated as chlorophyll *a* concentrations), and during the wet season, when habitat productivity and allochthonous carbon input are greatest (e.g., Stepanauskas et al. 2000, Caliman et al. 2010), *P. januarius* should have greater adult and embryo fat content, lean weight, reproductive investment, fecundity (coupled with smaller offspring), and body size (Reznick and Yang 1993, Riesch et al. 2016a), as well as deeper bodies (Spoljaric and Reimchen 2007).

Prediction 6 (pH-effect): Differences in pH cause physiological stress in various aquatic organisms, especially during early life-stages (Crespel et al. 2017). While its effects on poeciliid phenotypes have seldom been studied, higher pH levels appear to be linked to reduced body size and fecundity (Riesch et al. 2015, Jourdan et al. 2016).

Materials and methods

Study system

We sampled *P. januarius* in four lagoons in the Parque Nacional da Restinga de Jurubatiba, Brazil, a protected area that features several coastal lagoons separated from the sea by narrow (50–100 m) strips of sand (Fig. 2.1), between July 2011 and July 2012. During this period, two lagoons were sampled twice and the other two three times (Tables A.1, A.2), during both the wet (January) and dry season (July). In the park, some smaller lagoons can dry up during prolonged drought years, and connections between lagoons during exceptionally wet years are rare (A. Petry, personal observation). However, the four lagoons studied here are temporally stable (i.e., there is no record of them drying up in years of very low precipitations) and are isolated from each other, thus they can be treated as evolutionarily independent replicates (Araújo et al. 2014).

The fish were sampled using seine nets, that were pulled along the sandbar (i.e., the strip of sand that divides the lagoon from the ocean) in three, 30 m long transects along the longest axis of the lagoon. During sampling, most of the fish present in the area were captured independently of species, thus we were able to note the presence of piscivorous fishes such as *Hoplias malabaricus* and *Hoplerythrinus unitaeniatus* (see Araújo et al. 2014) as well as potential competitors (mainly *P. vivipara* and the anablepid *Jenynsia darwini*). Sampled *P. januarius* were immediately euthanized using clove oil, fixed in 10% formalin and preserved in 70% ethanol. During each sampling event we also measured dissolved oxygen, salinity, and temperature using a YSI-85-hydrometer, and collected water samples that were used in the laboratory to quantify pH using a Digimed DM-20 pH-meter, and chlorophyll *a* by filtering them through fiber-glass filters (GF/C Whatman), extracting chlorophyll *a* with 90% ethanol, and quantifying absorption at 665 nm using a spectrophotometer. These environmental parameters were measured once per sampling event in an intermediate point along the longest axis of the lagoon, as they are relatively uniform within each lagoon (A. Petry, unpublished data). Nevertheless, we found that they varied between lagoons, as well as between successive sampling events within the same lagoon (see Table A.3).

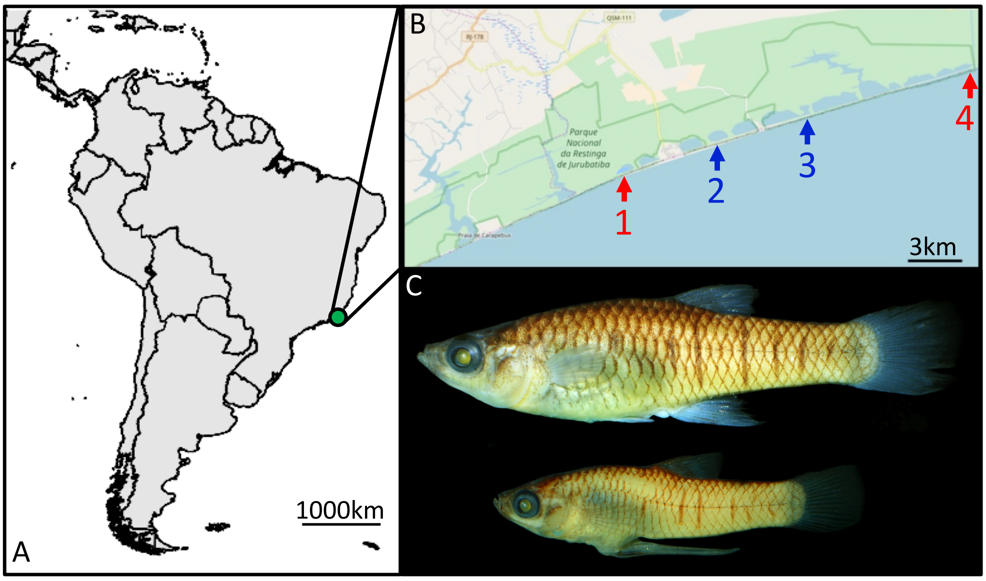

Figure 2.1. A Study area in the state of Rio de Janeiro; map created with the R-package maps (Becker et al. 2017). B Locations of the lagoons; map created using OpenStreetMap (https://www.openstreetmap.org, accessed on 14 December 2017): (1) Bezerra, (2) Maria Menina, (3) Catingosa and (4) Pitanga. Lagoons with piscivorous predators are presented in red and those without predators in blue. C Female (top) and male (below) adult *Phalloptychus januarius*.

Body-shape analysis

We analysed *P. januarius* body-shape variation by using geometric morphometric analyses (Rohlf and Marcus 1993, Adams et al. 2004). For each sampling event, we randomly selected a subset of 20 females and 0-10 males (Tables A.1, A.2), in order to avoid bias towards any specific size class, for a total of 200 females and 56 males. Following well-established protocols (Riesch et al. 2016a) we took standardised lateral photographs of each individual using a Canon EOS 1200 D digital camera (Canon Inc., Tokyo, Japan) with a fixed 60mm macro lens mounted on a copy stand. All pictures were collated together into a .TPS file using tpsUtil32 (Rohlf 2016a), and, using tpsDig232 software (Rohlf 2016b), we carefully positioned 15 landmarks on each fish picture (following Riesch et al. 2016; see also Appendix A and Fig. A.1 for a detailed description of the location of each landmark). The coordinates of the landmarks were analysed using relative warp analysis (Zelditch et al. 2012) using tpsRelw32 (Rohlf 2016c). This software first calculates the average shape across all specimens, or *reference configuration*, then aligns them in order to remove the effects of rotation, translation and scale. It then performs a principal component analysis on the distances between each landmark and the corresponding landmark of the reference configuration, across all specimens; these principal components are called *relative warps* (Rohlf 2015). We retained two relative warps that accounted for 90.24% of the cumulative variance (Table A.4) and were used as shape variables in all subsequent analyses. As part of the analysis, the program automatically calculates also the centroid size of each individual (i.e., the square root of the sum of the squared distances of each landmark from their centroid), which we used as a covariate in subsequent analyses to account for differences in body size between individuals.

Life-history traits and proxies

We dissected 200 pregnant females and 44 mature males following well-established protocols to assess life-history traits and life-history proxies (Reznick and Endler 1982, Riesch et al. 2016a). We assessed female fecundity (number of developing offspring), offspring lean weight [mg] (we refer to it as weight instead of mass in order to remain consistent with the terminology used in the published literature), offspring fat content [%], female reproductive allocation (RA [%], i.e., total offspring dry weight divided by the sum of offspring plus somatic tissue dry weight), and male gonadosomatic index (GSI [%], i.e., testis dry weight divided by the sum of somatic plus reproductive tissue dry weight). Moreover, as proxies for investment into growth and maintenance, we measured male and female standard length (SL [mm]), lean weight [mg] and fat content [%].

Embryonic traits need to be considered relative to the embryo's developmental stage. We therefore assessed the developmental stage of each embryo following Riesch et al. (2011), with stages ranging from 2 (fertilized oocytes) to 50 (ready-to-be-born embryos). To account for superfetation and high levels of matrotrophy in *P. januarius* (Pollux and Reznick 2011), we estimated offspring size at birth for each individual female by regressing embryo weight against embryonic stage of development. Based on R^2, cubic regressions were the best-fitting model. Furthermore, we calculated the Matrotrophy Index (MI; Reznick et al. 2002a) for each individual female as the ratio between two extrapolated values: offspring weight at birth and weight of the unfertilized egg (stage 0). However, cubic regressions cannot provide accurate results for females with fewer than 5 different embryo stages. To avoid missing data values, we calculated mean offspring weight at birth and mean MI for each population and used these values for this particular subset of females ($N = 15$).

To meet statistical assumptions of normality of residuals, we $\log_{10}$-transformed (SL, adult lean weight, offspring weight at birth and MI), square root-transformed (fecundity), or arcsine (square root)-transformed (GSI, RA, adult and embryo fat contents) all variables. We subsequently z-transformed all variables to obtain unit-free variables of similar scale.

Statistical analyses

Population differences and seasonal variation

We compared body size (SL) between lagoons using ANOVA with ‘sex’, ‘lagoon’, and ‘sampling-date-nested-within-lagoon’ [henceforth ‘date(lagoon)’—to account for multiple sampling in each lagoon] as factors. We ran MANCOVA on body shape with ‘centroid size’ as a covariate and including the aforementioned factors. We analysed male and female life-history traits and proxies in two separate MANCOVAs while including ‘SL’ as a covariate, and ‘lagoon’ as well as ‘date(lagoon)’ as factors. We initially included all interaction terms, and subsequently removed terms with $P > 0.1$. We approximated F-values using Wilks’ Lambda and estimated relative effect strengths using partial eta squared (η^2). We corrected alpha-levels for multiple comparisons as $\alpha' = \alpha$/number of comparisons (Table A.5).

Relative effects of different environmental parameters

We evaluated the relative importance of each environmental variable in driving phenotypic divergence by employing a model averaging approach (Burnham and Anderson 2002). We first screened all environmental variables for any evidence of multicollinearity by running bivariate correlations between salinity, temperature, dissolved oxygen, chlorophyll *a*, and pH. We did not find any significant correlations across all environmental variables (Pearson's correlation, $P > 0.100$ in all cases). We then corrected traits for which significant sex- and body size-effects were uncovered (*post-hoc* ANCOVAs, Table A.5) by regressing RW1 and RW2 against 'centroid size' and 'sex', female lean weight, fecundity and superfetation against 'SL', and male lean weight against 'SL'. We finally used residuals as dependent variables in identical, trait-wise global linear models with 'presence of predators' coded as a factor and salinity, temperature, dissolved oxygen, chlorophyll *a* as well as pH as covariates. 'Lagoon' was included as a random factor to account for the repeated sampling in each lagoon. Using the *dredge* function in the MuMIn package in R (Barton 2011), we performed model selection by fitting all possible model permutations and ranking them using Akaike Information Criteria corrected for small sample size (AIC*c*). As there was no one-best-model in all cases, we selected a subset of most informative models with $\Delta \text{AIC}c \leq 7$ from the model pool (Burnham et al. 2011). Using this subset, we performed model averaging using the *model.avg* function. Model averaging uses information criteria such as AIC*c* to assess the predictive power of explanatory variables (relative importance values, hereafter *RIV*; i.e., the cumulative Akaike weights across all subset models that contain the variable), and to obtain averaged parameter estimates (model-averaged coefficients; hereafter b_{MA}) from a set of models (Burnham and Anderson 2002, Grueber et al. 2011). Statistical analyses were conducted using IBM® SPSS® Statistics v.21 (IBM Corp. 2012; ANOVAs and MANCOVAs) and R (R Development Core Team 2018; model averaging).

Results

Sexual dimorphism and phenotypic variation within and between lagoons

Body size varied significantly between sexes (ANOVA, $F_{1,230} = 658.98$, $P < 0.001$), lagoons ($F_{3,230} = 6.87$, $P < 0.001$), and sampling events ($F_{6,230} = 2.71$, $P = 0.015$). While males were smaller than females, sexual size dimorphism varied amongst lagoons (‘sex × lagoon’; $F_{3,230} = 3.94$, $P = 0.009$; Tables A.1, A.2).

Similarly, the strongest effect in the MANCOVA analysing body-shape variation reflected differences between the sexes (Table 2.1), followed by allometric effects (‘centroid size’), differences among lagoons and across repeated samplings. However, also the interactions of ‘sex × lagoon’, ‘sex × date(lagoon)’ and ‘centroid size × lagoon’ were significant (Table 2.1). *Post-hoc* univariate models (with corrected alpha-levels: $\alpha' = 0.025$) found significant allometric effects and sex-differences for both RW1 and RW2 (all $P \leq 0.001$), while ‘lagoon’ had a significant effect only on RW1 ($P < 0.001$) and ‘date(lagoon)’ on RW2 ($P = 0.002$; Table A.5*a*). The sex-effect can be explained by the presence of the gonopodium in males and a generally more anteriorly-positioned anal fin compared to females (Fig. 2.2), while differences between lagoons and across seasons within lagoons mainly reflect differences in body depth and roundness. Furthermore, body shape scaled differently with body size across lagoons (‘centroid size × lagoon’-effect), and there were differences among lagoons in the direction and extent of sexual dimorphism (‘sex × lagoon’-effect; Fig. A.2). Seasonal variation affected both sexes differently [‘sex × date(lagoon)’-effect; Table 2.1].

Both male and female life-history traits and proxies were significantly affected by body size (SL), but we also found significant spatial (‘lagoon’-effect) and temporal [‘date(lagoon)’-effect] differences, as well as a significant effect of ‘SL × lagoon’, indicating that some traits scaled differently between lagoons (Table 2.2). For female traits, we further uncovered a significant effect of ‘SL × date(lagoon)’, indicating that the extent to which traits scaled with female body size differed in time. *Post-hoc* univariate models (corrected alpha-levels were $\alpha' = 0.006$, and $\alpha' = 0.017$ for analyses of female and male traits, respectively) revealed that body size had significant positive associations with male and female lean weight, fecundity, and superfetation (Table A.5*b*, *c*). For females, lean weight, fecundity, estimated offspring size at birth, MI, superfetation, embryo fat content and RA all differed between lagoons, while for males only lean weight and GSI did. Temporal variation within lagoons was uncovered for all female traits except embryo fat content, but only for GSI in males (Table A.5*b*, *c*).

The interaction of ‘SL × lagoon’ had a significant effect on male fat content (Table A.5*c*): bigger males had higher fat contents in low-salinity

(Bezerra and Pitanga) but lower fat contents in high-salinity lagoons (Catingosa and Marina Menina).

Finally, the interaction ‘SL × date(lagoon)’ significantly affected female fecundity, superfetation, and RA (Table A.5*b*). These differences appear to be mostly linked to yearly variation, as fecundity and superfetation increased strongly with SL in July 2011 and January 2012, but weakly in July 2012 (Fig. A.3; A.4). Larger females also had greater RA in July 2011 and January 2012, but this relationship was reversed in July 2012 (Fig. A.5).

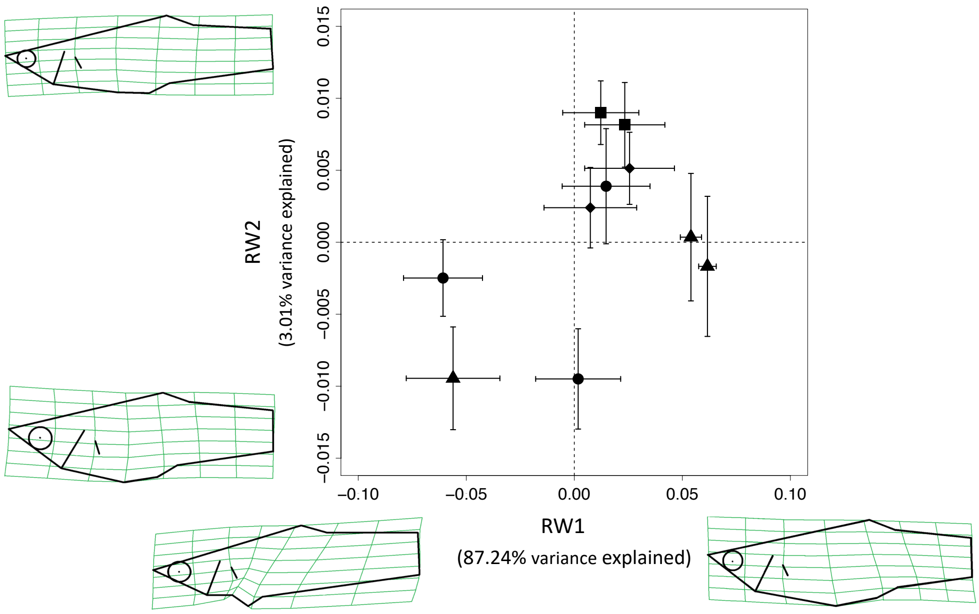

Figure 2.2. *P. januarius* body-shape variation along RW1 and RW2 (mean ± SE). Bezerra: squares, Catingosa: triangles, Maria Menina: circles, Pitanga: diamonds; multiple symbols represent the repeated sampling in each lagoon. Thin-plate spline transformation grids visualize morphological variation across the two relative warps, whereby RW1 mainly separates males (left) from females (right), whereas RW2 describes differences in body roundness and depth.

Table 2.1. Multivariate analysis of covariance (MANCOVA) on body shape divergence of *P. januarius* that were repeatedly collected in four coastal lagoons. Statistically significant effects are highlighted in bold.

Factors	*F*	Degrees of Freedom	*P*	Partial η^2	Relative η^2
Centroid size	16.622	2, 231	**< 0.001**	0.126	0.333
Sex	70.283	2, 231	**< 0.001**	0.378	1.000
Lagoon	4.452	6, 462	**< 0.001**	0.055	0.146
Date(lagoon)	2.426	12, 462	**0.005**	0.059	0.156
Sex × lagoon	5.004	6, 462	**< 0.001**	0.052	0.137
Sex × date(lagoon)	2.314	8, 462	**0.019**	0.039	0.103
Centroid size × lagoon	4.261	6, 462	**< 0.001**	0.052	0.137

Table 2.2. Multivariate analysis of covariance (MANCOVA) on (*a*) female and (*b*) male life-history traits and proxies of *P. januarius* from four coastal lagoons. Statistically significant effects are highlighted in bold.

Factors	*F*	Degrees of Freedom	*P*	Partial η^2	Relative η^2
(*a*) Female life histories					
SL	114.898	8, 173	**< 0.001**	0.842	1.000
Lagoon	10.167	24, 502	**< 0.001**	0.318	0.427
Date(lagoon)	5.224	48, 855	**< 0.001**	0.190	0.243
SL × lagoon	1.831	24, 502	**0.010**	0.078	0.142
SL × date(lagoon)	1.88	48, 855	**< 0.001**	0.079	0.092
(*b*) Male life histories					
SL	39.67	3, 30	**< 0.001**	0.799	1.000
Lagoon	5.43	9, 73	**< 0.001**	0.340	0.426
Date(lagoon)	3.20	12, 80	**0.001**	0.293	0.367
SL × lagoon	2.739	9, 73	**0.008**	0.210	0.263

Effects of environmental parameters on trait divergence

Model averaging revealed that most environmental parameters affected at least one life-history trait or proxy, while there were no significant effects of environmental variation on body-shape traits (Table 2.3). Even though there were additional trends that conformed (or were sometimes opposite) to our *a priori* predictions, we will mainly outline significant effects here.

Effects of predation

The effect of predation was generally in the predicted direction (Table 2.3). In high-predation lagoons females were smaller (SL) and both males and females had lower lean weight than in low-predation ones (Fig. 2.3). Moreover, in high-predation environments, females had higher levels of superfetation.

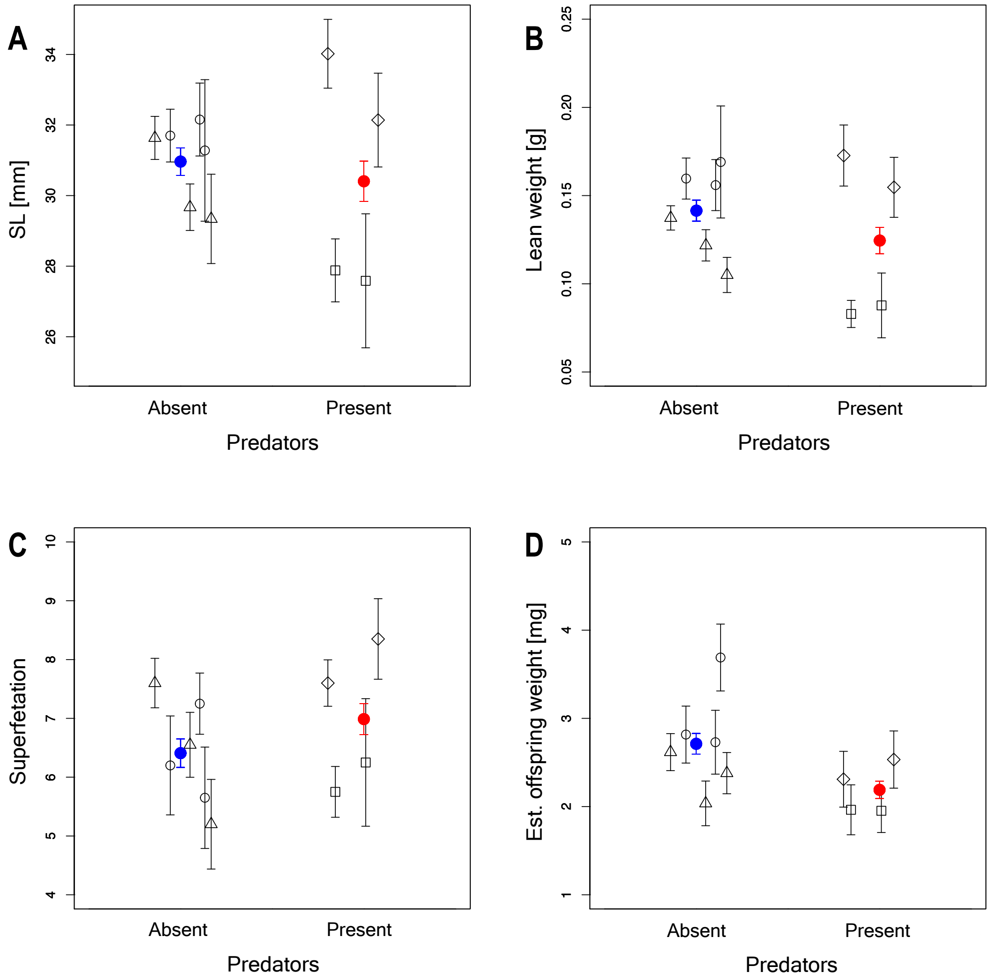

Figure 2.3. Mean ± SE of female life-history traits and proxies (A: SL, B: adult lean weight, C: superfetation, D: estimated offspring weight at birth) in populations exposed to different predation regimes. Bezerra: squares, Catingosa: triangles, Maria Menina: circles, Pitanga: diamonds; multiple symbols reflect the repeated sampling in each lagoon. Red symbols represent the overall mean across high-predation lagoons, while blue represents the overall mean across low-predation lagoons.

Effects of salinity

Most effects of salinity on life-history traits and proxies were significant (Table 2.3). Higher salinity was associated with larger female body size (SL), increased lean weight in both sexes, and increased offspring size at birth, but also with lower levels of superfetation and female reproductive investment (RA; Fig. 2.4).

Effects of water temperature

Higher temperatures were associated with lower female fecundity and RA, and less superfetation, while males showed a lower GSI and embryos an increased body fat content (Fig. 2.4).

Effects of dissolved oxygen, chlorophyll *a* and pH

Dissolved oxygen (DO) and pH were overall the strongest predictors of phenotypic differentiation (based on *RIV*s). Increases in DO were associated with decreased fecundity, MI, and superfetation, as well as decreased investment into reproduction in both sexes (i.e., RA and GSI). Embryo fat increased with increasing DO (Table 2.3).

Chlorophyll *a*, on the other hand, did not significantly affect any life-history trait, as all model-averaged coefficients bounded zero (Table 2.3).

Finally, pH had strong positive associations with female SL, male and female lean weight, female fat content as well as RA and GSI (Table 2.3).

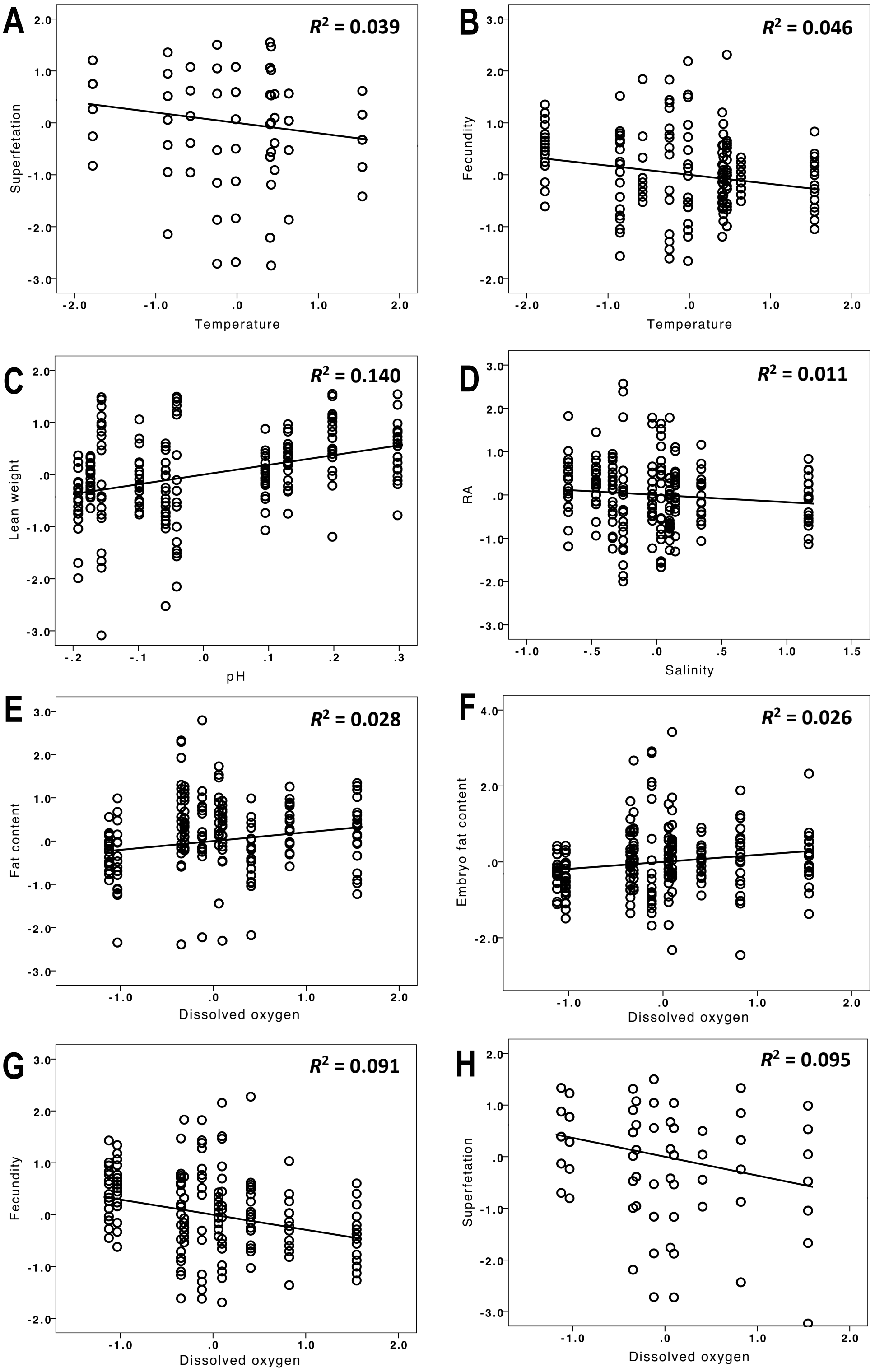

Figure 2.4. Partial regression plots of female phenotypic variation due to different environmental variables. The plots (and regression lines) are derived from the global model used for the model averaging analysis and data points represent residuals corrected for all the other terms in the model. (A) Temperature effect on superfetation and (B) on fecundity; (C) variation in lean weight due to pH; (D) salinity-effects on RA; (E) dissolved oxygen-effect on adult fat content, (F) on embryo fat content, (G) on fecundity, and (H) on superfetation.

Table 2.3. Summary results from model averaging on phenotypic divergence. Reported are relative importance values (*RIV*) and model averaged coefficients (b_{MA}) for females; values in parenthesis represent the results of male traits. Predictors with significant effects are highlighted in bold.

		Predators	O_2	pH	Salinity	Temperature	Chlorophyll *a*
RW1	*RIV*	0.50	0.98	0.34	0.82	0.38	0.98
	b_{MA}	-0.010	-0.004	0.003	-0.001	-0.001	0.001
RW2	*RIV*	0.37	0.52	0.75	0.35	0.80	0.27
	b_{MA}	-0.007	-0.002	-0.007	0.001	0.001	0.001
SL	*RIV*	**0.94** (0.27)	0.23 (0.25)	**0.94** (0.23)	**0.94** (0.31)	0.17 (0.19)	0.63 (0.22)
	b_{MA}	**-3.395** (0.405)	-0.040 (0.180)	**1.762** (0.269)	**0.067** (0.014)	-0.027 (0.016)	-0.007 (-0.002)
Lean weight	*RIV*	**0.90** **(0.65)**	0.76 (0.13)	**0.90** **(0.65)**	**0.90** **(0.79)**	0.23 (0.15)	0.30 (0.18)
	b_{MA}	**-0.597** **(-1.302)**	0.036 (-0.017)	**0.293** **(0.674)**	**0.027** **(0.038)**	0.001 (0.001)	0.001 (0.001)
Fat content	*RIV*	0.53 (0.22)	0.46 (0.19)	**0.88** (0.24)	0.64 (0.21)	0.56 (0.20)	0.94 (0.25)
	b_{MA}	-2.123 (0.144)	0.217 (0.024)	**0.893** (0.183)	0.060 (-0.005)	-0.135 (0.023)	0.015 (-0.007)
Fecundity	*RIV*	0.50	**0.98**	0.46	0.52	**0.97**	0.96
	b_{MA}	0.725	**-0.252**	0.299	-0.017	**-0.092**	0.009
Est. offspring weight at birth	*RIV*	0.57	0.55	0.14	**0.98**	0.38	0.28
	b_{MA}	-0.484	-0.077	-0.137	**0.051**	0.049	-0.002
MI	*RIV*	0.38	**0.62**	0.22	0.81	0.39	0.41
	b_{MA}	-0.259	**-0.125**	-0.084	0.030	0.010	0.005
Superfetation	*RIV*	**0.96**	**0.96**	0.23	**0.96**	**0.96**	0.91
	b_{MA}	**1.253**	**-0.288**	-0.059	**-0.066**	**-0.259**	0.008
Embryo fat content	*RIV*	0.43	**0.99**	0.30	0.27	0.44	0.99
	b_{MA}	0.466	**0.197**	-0.056	0.002	0.043	0.018
RA (GSI)	*RIV*	0.09 (0.51)	**0.97** **(0.99)**	**0.97** **(0.99)**	**0.97** (0.39)	**0.97** **(0.99)**	0.35 (0.99)
	b_{MA}	0.119 (-0.842)	**-0.527** **(-0.607)**	**0.581** **(1.008)**	**-0.053** (-0.022)	**-0.253** **(-0.404)**	-0.003 (0.033)

Discussion

We uncovered strong phenotypic differentiation in *P. januarius* between sexes, lagoons, and sampling seasons. Most environmental gradients considered affected *P. januarius* phenotypes to some extent. However only some of the patterns followed out *a priori* predictions, and the strongest drivers of phenotypic differentiation were not predation or salinity, bu unexpectedly DO and pH.

Sexual dimorphism and phenotypic variation within and between lagoons

Patterns of sexual dimorphism in phenotypic traits are congruent with those described for other poeciliids (Bisazza 1993). Seasonal variation of those traits is also well documented in numerous taxa, including poeciliid fishes (Reznick 1989, Abney and Rakocinski 2004, Almeida-Silva and Mazzoni 2014). The direction of seasonal changes in individual life-history traits and proxies, however, was not the same across lagoons. Nonetheless, significant differences among lagoons persisted even when controlling for temporal variation, and the relative importance of the lagoon-effect (estimated using partial η^2) was consistently greater than temporal differences, while remaining relatively low.

One possible explanation is that, due to our sampling method, the fish sampled might have been of a certain phenotype along the shy/bold continuum, and this might have in turn influenced their life histories or body shape. Different fishing gears have been shown to impact fish phenotypes (Diaz Pauli and Sih 2017). In *P. reticulata*, for instance, seines appear to catch preferentially shyer individuals, as bolder ones are better at escaping (Diaz Pauli et al. 2015). While it is not known whether boldness and other phenotypic traits are correlated in *P. januarius*, such associations have been found in other poeciliid species, such as *G. holbrooki* (Wilson et al. 2010) and *Brachyrhaphis* spp. (Ingley et al. 2014), and might, therefore be important here. At present, however, we lack empirical data to properly address this problem, and we call for future studies to focus on correlations between multiple phenotypic trait suites in this species.

Nevertheless, the magnitude of lagoon-specific differences reported here are similar to what was previously reported for *P. vivipara* from the same region (Gomes-Jr and Monteiro 2007, Araújo et al. 2014, Rius et al. 2019). This further supports the notion that environmental heterogeneity in these lagoons facilitates strong phenotypic differentiation. In the following, we will explore which environmental variables might be underlying these phenotypic differences both among and within lagoons.

Effects of environmental parameters on trait divergence

In our model averaging analysis, we found support only for some of our *a priori* predictions. One of the possible reasons for the relative lack of responses to environmental variation in the predicted directions might stem from the fact that we measured all environmental parameters at the same time as we sampled the fish. While the sampled lagoons are known to be temporally stable (i.e., there are no records of them drying up in drought years), this habitat is known to experience strong environmental variation between different seasons and years (Caliman et al. 2010). For instance, between July 2011 and January 2012, Pitanga went from being a highly-saline lagoon to be the one with the lowest salinity, and, albeit at a much smaller scale, the other lagoons also varied in their salinity between sampling events (Table A.3). It is therefore possible that there might be a time lag between environmental change and phenotypic responses that leads to a carry-over effect, i.e., that fish might be adapted to conditions experienced some generations ago (Harrison et al. 2010). On the other hand, we cannot currently exclude the possibility that most of the observed variation might also reflect phenotypic plasticity rather than evolved (heritable) differences among populations (however, see Pfennig et al. 2010 for the importance of plasticity in diversification), and the relative importance of either of these processes will have to be investigated in future studies.

Effects of predation

Predation had a significant effect on several life-history traits and proxies, but unexpectedly, did not have a significant effect on body-shape divergence. In support of our prediction 1, females from high-predation lagoons were smaller. Contrary to prediction 1, however, male body size was larger and male and female lean weight lower in high-predation lagoons. Where patterns followed the predicted direction, this matched patterns of divergence reported for *P. vivipara* from the same and additional lagoons (Neves and Monteiro 2003, Gomes-Jr and Monteiro 2007, Araújo et al. 2014, Rius et al. 2019) and for other poeciliids inhabiting environments with different predation intensity (e.g., *Brachyrhaphis episcopi*: Jennions and Telford 2002; *Gambusia* spp.: Riesch et al. 2015; *P. reticulata*: Reznick and Endler 1982).

One pattern we did not specifically predict was that females increased the level of superfetation as predation intensity increased. Nonetheless, this is in line with previous work and matches the hypothesis that environments with high adult mortality should select for increased rates of superfetation (Zúñiga-Vega et al. 2010; see also Travis et al. 1987). Increased superfetation in response to high predation levels has also been reported in another superfetating poeciliid (*Phalloceros harpagos*; Gorini-Pacheco et al. 2017).

Predation did not have strong associations with body shape, contrary to most previous studies (Neves and Monteiro 2003, Araújo et al. 2014). One potential explanation is that selection from predation could be weaker in *P. januarius* than in the two other species of livebearing fishes present in these lagoons (*P. vivipara*, *J. darwini*), which have larger body sizes (Araújo et al. 2014). Predators of livebearing fishes tend to preferentially target large individuals as prey (e.g., Trexler et al. 1994, Johansson et al. 2004, Tobler et al. 2007) and could selectively prey on *P. vivipara* and *J. darwini* rather than on *P. januarius*. While we currently lack empirical data, this could result in stronger body-shape divergence in the former species (Araújo et al. 2014).

Effects of salinity

Alongside predation, salinity had previously been characterised as the defining selective agents in this system (Gomes-Jr and Monteiro 2007, Araújo et al. 2014). Salinity indeed showed a number of statistical associations with several traits. However, other environmental variables (i.e., pH and oxygen content) were of similar importance, and it is important to note that *P. januarius* inhabits lagoons with a narrower salinity gradient than *P. vivipara*. Nonetheless, in highly-saline lagoons, females were larger (SL), males and females had an increased lean weight, and offspring size at birth was greater (in agreement with our prediction 2). On the other hand, RA decreased at higher salinity (contrary to prediction 2), and superfetation was reduced. This mixed pattern of results supporting and opposing *a priori* predictions is congruent with a recent meta-analysis that did not find salinity to result in strong, consistent patterns of divergence in offspring size and fecundity across poeciliid species (Moore et al. 2016). Yet, in our present study, the effects of salinity were strong, and similar patterns were reported from other poeciliids (e.g., Alcaraz and García-Berthou 2007, Martin et al. 2009). We argue that salinity-effects may be more system-specific than those of other environmental variables (Moore et al. 2016).

Effects of temperature

Contrary to prediction 3, under warmer conditions, fish showed a lower (not higher) fecundity, superfetation, GSI and RA when compared to samples obtained at colder conditions, while body size (SL and lean weight) did not show strong associations with temperature, and the trend for female body fat was even negative. Previous laboratory experiments on *Gambusia affinis* found high temperatures to result in increased growth (and therefore body size), fecundity and reproductive investment (Vondraceck et al. 1988). Moreover, warmer seasonal temperatures were associated with increased fecundity and greater total brood mass in *Gambusia puncticulata* (Abney and Rakocinski 2004). Our results suggest a strong decrease in reproduction during the warmer Brazilian summer months (i.e., the wet season). This is congruent with patterns reported for Trinidadian guppies, which decreased fecundity and reproductive allocation during the warm months of the rainy season, and then increased both during the colder dry season (Reznick 1989). While it is difficult to disentangle the effects of temperature from other, potentially confounding factors we did not quantify, we suspect that seasonal effects in our present study system might partially reflect antagonistic interactions between the three poeciliid species inhabiting these lagoons: under more favourable conditions (i.e., during the wet season) *P. januarius*, being the smallest of the three species, might be outcompeted by the larger two species, while they might be better competitors under low-resource conditions (during the dry season). Similar dynamics have been reported by Winemiller (1989) for other tropical fish assemblages. We are currently lacking empirical data to properly address this issue, so we call for more research into the effects of seasonal variation in temperature regimes on competitive interactions between the three species, but also on phenotypic divergence in general.

Effects of dissolved oxygen, chlorophyll *a* and pH

DO had one of the strongest effects on phenotypic differentiation, but patterns did not match our prediction 4, as, for example, in low-oxygen environments, females had higher fecundity, while we did not find the expected response of increased head size. One possible explanation is that variation in DO in these coastal lagoons was not big enough to elicit the predicted hypoxia-related responses. In our samples, DO levels ranged from 5.01 to 9.27 mg O_2/L, always far above the level below which aquatic environments are considered hypoxic (2 mg O_2/L; Vaquer-Sunyer and Duarte 2008, Chapman 2015; although we did not quantify the full diurnal range of DO variation). Nonetheless, even these nuanced changes in DO were associated with several prominent shifts in life-history traits: both males and females drastically reduced their investment into reproduction under elevated oxygen concentrations, with lower GSI in males and lower fecundity, superfetation, MI and RA in females, while embryo fat content was increased. While the evolutionary effects of hypoxic conditions have been studied in several species of fish (Chapman 2015), few studies have directly investigated the effects of smaller, more gradual differences in DO. Similar to our results, DO and fecundity were inversely correlated in *G. holbrooki* from rice fields in Portugal (Cabral and Marques 1999), whereas high oxygen levels appear to be linked to an increased fecundity in *G. hubbsi* (Riesch et al. 2015). We cannot, however, exclude the possibility that some of the strong phenotypic responses we detected in response to relatively subtle differences in DO at the point of sampling reflect responses to greater diurnal fluctuation, which we did not quantify. Clearly, more research on how exactly DO impacts fish life histories, in particular their reproductive traits, is needed. Nonetheless, it is also possible that the effects reported here are indirect effects—mediated by predation—as the two piscivorous fishes in this system are tolerant to low oxygen conditions (Petry et al. 2013). Low DO might increase predation risk (e.g., when prey individuals spend more time in certain oxygen-rich microhabitats), which would align well with the uncovered patterns of divergence in life-history traits (e.g., Reznick et al. 1990, 2002b, Riesch et al. 2015).

The effects of chlorophyll *a* levels—our proxy for autochthonous habitat productivity—on *P. januarius* phenotypes were all not significant, contrary to our prediction 5. Previous experimental studies on livebearing fishes showed that variation in food availability can elicit strong phenotypic responses (e.g., *Poecilia mexicana*: Riesch et al. 2016b; *P. reticulata*: Reznick 1989, Reznick and Yang 1993), as predicted by life-history theory (e.g., Reznick et al. 2002b). Still, in our case, the presence of other environmental factors, such as predation (Grether et al. 2001) or seasonal differences (see previous paragraph), might overshadow or partially counteract the direct effects of food availability, leading to the lack of significant differences.

Finally, we found that females increased SL and fat content, and both sexes increased their lean weight and investment into reproduction (RA and GSI) with increasing pH. These results are, if anything, contrary to our prediction 6, but it remains difficult to discuss our findings in a broader conceptual context, as potential effects of variation in pH on variation of life-history traits and proxies in fishes are understudied (Nelson 2015). Differences in pH are known to cause physiological stress (EIFAC 1969, Crespel et al. 2017) and have been recognised as one of the main factors shaping fish communities in Indian rivers (Sharma et al. 2017). It seems likely that pH-levels play underestimated roles in shaping phenotypic differentiation between populations and warrant increased attention in future experimental studies.

Conclusions

Patterns of phenotypic divergence in *P. januarius* only partially conformed to our *a priori* predictions, and several traits even exhibited patterns opposite to the predicted directions. We argue that multifarious selection pressures, experienced by our study species in different lagoons and at different points in time within specific lagoons, explain this pattern. Furthermore, we cannot exclude that other environmental factors, that were not considered here, might also be acting in this system, further impacting the phenotypic patterns that we found. Organisms are usually exposed to a multitude of environmental selective forces that act on the same or different components of the whole phenotype at any given time, and each of these selective forces is likely to have a different relative impact on organismal fitness. This should lead to a mosaic of organismal responses, with some selective forces cancelling each other out, some acting synergistically, while yet other selective forces might be so strong that they overrule potential responses to others (Riesch et al. 2018). This could result in somewhat spurious patterns of phenotypic responses to specific environmental variables (e.g., pH or temperature in our present study), including patterns opposite to those predicted if each environmental variable were to act alone. Thus, more experimental work on responses to several interacting environmental factors is clearly needed. Our study also reveals that our understanding of how exactly some of the environmental variables assessed here might shape organismal responses is still limited, highlighting the need for more experimental work on the effects of as yet understudied environmental factors in driving phenotypic diversification.

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Data Accessibility

Data is available via the Royal Holloway Figshare Data Archive at doi:0.17637/rh.8948951

Conflict of Interest

The authors declare that they have no conflict of interest.

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Chapter 3: A century later: adaptive plasticity and rapid evolution contribute to geographic variation in invasive mosquitofish

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Highlights

- Mosquitofish (*Gambusia holbrooki*) invaded Europe less than 100 years ago
- Population genetics suggest single introduction from the same source population
- Climate shapes phenotypic diversification across their European invasive range
- Common-garden rearing suggests a minor contribution of genetic evolution
- Plasticity of morphological and life-history responses largely maintained

Abstract

One century after their introduction to Europe, eastern mosquitofish (*Gambusia holbrooki*) represent a natural experiment to disseminate the relative contributions of adaptive plasticity and rapid evolutionary change in creating large-scale geographic variation in phenotypes. We evaluated the population-genetic structure and invasion history based on allele length polymorphisms of 15 nuclear microsatellites, which we quantified for N = 660 individuals from 23 populations sampled in 2013 across the invasive range of *G. holbrooki* in Europe. We analysed body-shape and life-history variation in N = 1,331 individuals from 36 populations, sampled in 2013 and 2017, and tested heritability of phenotypic differences in a subset of four populations using a common-garden experiment. The genetic structure of wild-caught individuals suggested a single introduction for all European mosquitofish, which were genetically impoverished compared to their native counterparts. We found some convergent patterns of phenotypic divergence across native and invasive climatic gradients (e.g., increased body size in colder/more northern populations); however, several phenotypic responses further varied between sampling years, pointing towards plastic phenotypes. Our analysis of common-garden reared individuals confirmed high levels of plasticity, as no phenotypic traits showed significant broad-sense heritability.

Our results highlight the importance of phenotypic plasticity in invasive species during range expansions.

Key-words

Biological invasion, range expansion, body shape, life histories, heritability, *Gambusia holbrooki*

Introduction

The colonization of new environments has important ecological and evolutionary consequences (Phillips et al. 2010). Following range expansions, species often encounter novel environmental conditions (Marques et al. 2018) to which they must rapidly respond to avoid local extinction (Reznick and Ghalambor 2001). Organisms can respond to environmental variation either through phenotypic plasticity (Ghalambor et al. 2007, Lande 2015)—i.e., the ability to alter their phenotypes with unchanged genotypes (West-Eberhard 2003)—or rapid evolutionary change (Reznick et al. 2019). In several cases, our knowledge of the mechanisms underlying phenotypic variation remains limited (Kruuk et al. 2003, Merilä and Hendry 2014), as it can be difficult to distinguish between plastic responses and rapid evolutionary change (Merilä and Hendry 2014). Moreover, phenotypic plasticity can influence rates of evolutionary change, either promoting or retarding genetic evolution (Levis and Pfennig 2016, Fox et al. 2019), and also the level of phenotypic plasticity itself (i.e., reaction norms) can be differently selected for (DeWitt and Schneider 2004).

Invasive species provide unplanned 'natural experiments' on environmentally-induced phenotypic diversification (Mooney and Cleland

2001, Sax et al. 2007, Whitney and Glaber 2008, Bock et al. 2015, Colautti and Lau 2015, Jourdan et al. 2019) and may provide invaluable insights into the relative contributions of plasticity and contemporary evolution following the colonization of new environments (Hendry 2015). Biological invasions often occur over large temporal and geographical scales, sometimes spanning entire continents (Rice and Sax 2005). They allow comparisons between native and invasive ranges, or between replicated invasion events (Colautti et al. 2009, Kelly 2019). Moreover, if the location and timing of an invasion event is known (e.g., Beckenbach and Prevosti 1986, Rosecchi et al. 2001, Bucharova and Van Kleunen 2009), rates of phenotypic diversification can be quantified (Sax et al. 2007, Reznick et al. 2019).

The importance of phenotypic plasticity for creating phenotypic variation has been demonstrated in invasive plants [e.g., *Taraxacum officinalis*, Molina-Montenegro and Naya 2012; alligator weed (*Alternanthera philoxeroides*), Geng et al. 2007; see also Richards et al. 2006, Davison et al. 2011] as well as in invertebrates (springtails, Chown et al. 2007; *Daphnia limholtzi*, Dzialowski et al. 2003; *Littorina obtusata*, Trussell and Smith 2000), fish (*Gobio gobio* and *Pseudorasbora parva*, Rosecchi et al. 2001), and birds (Duncan et al. 2003). On the other hand, an increasing number of studies identified examples of adaptive evolution taking place within few generations after introduction (Mooney and Cleland 2001, Barrett et al. 2008, Dlugosh and Parker 2008, Prentis et al. 2008, Suarez and Tsutsui 2008, Reznick et al. 2019). For example, in the 20 years following the introduction of *Drosophila subobscura* from Europe to both North and South America (described as "*a grand experiment in evolution*" by Ayala et al. 1989), an adaptive cline in wing size was observed, similar to what had been described for the species' native distribution range (Huey et al. 2000, Gilchrist et al. 2004). Likewise, Johnston and Selander (1971) reported the evolution of a body size cline in invasive house sparrows (*Passer domesticus*) from a large geographical range in North America similar to the cline observed in the species' native range. Finally, within only 10-14 years following their introduction to several Caribbean islands, *Anolis* lizards evolved morphological adaptations to their new environments (Losos et al. 1997).

Here, we used the invasion of eastern mosquitofish (*Gambusia holbrooki*) across Europe to study the mechanisms underlying phenotypic differentiation over large geographic scales (> 2,000 km). These small fish of the poeciliid family are native to the United States of America (Pyke 2005) but have been introduced to Spain and Italy during the 1920s for mosquito control (Grapputo et al. 2006)—with varying degrees of efficacy (Kottelat and Whitten 1996, Pyke 2008, Azevedo-Santos et al. 2017)—and successfully colonised a wide range of environments. Mosquitofish are now widespread and common in Europe, ranging as far north as central France (Pyke 2005, Grapputo et al. 2006, Benejam et al. 2009). In their native range along the East Coast of the USA, mosquitofish exhibit latitudinal (climatic) clines, with larger body size, greater reproductive investment, smaller offspring and shallower bodies coupled with smaller heads in northern and colder climates compared to southern/warmer ones (Riesch et al. 2018). Phenotypic divergence due to environmental variation has also been observed in invasive *G. affinis* from China (Ouyang et al. 2018), and *G. holbrooki* from Europe; albeit in the latter case, fish were sampled across a relatively small geographical area within their invasive range (Benejam et al. 2009). In the closely related *Gambusia affinis*, Stockwell and Weeks (1999) found that rapid evolution of life-history traits occurred within 55-58 years after the founding of four populations in the USA. However, it is currently not known whether, across their invasive range, geographic variation in mosquitofish phenotypes is driven by (*a*) phenotypic plasticity or (*b*) rapid local adaptation (or a combination of both). Alternatively, (*c*) phenotypic variation may be a consequence of multiple introductions of phenotypically diverse fish from multiple source populations across their native range (but see Vidal et al. 2010, Sanz et al. 2013).

We sampled *G. holbrooki* across their invasive range in France, Italy and Spain and used population genetic methods to investigate the genetic structure of invasive populations and their invasion history. Furthermore, we analysed body-shape and life-history differentiation between wild-caught individuals from 36 different populations. We estimated broad-sense trait heritability in a subset of four populations that were raised under common-garden laboratory conditions for two generations. We aimed to answer the following questions: (1) What is the population genetic structure of European mosquitofish, and does it suggest a single or multiple introductions? (2) What is the extent of body-shape and life-history variation across the species' invasive range in Europe? (3) Which environmental factors (including climatic variation and habitat-specific environmental parameters) are associated with the observed patterns? (4) Are phenotypic differences due to plasticity or do they have a genetic basis?

Materials and methods

Sampling sites and environmental parameters

We sampled *Gambusia holbrooki* from 36 independent populations across Italy in May 2013, Spain and France in September 2013, and Italy and Spain in July/August 2017; in addition, four of these populations were samples in both years to provide a direct measure of temporal variation (Fig. 3.1; Table B.1). All fish were sampled using hand-held dip nets, immediately euthanized using clove oil, and preserved in 70% ethanol for subsequent analyses. After sampling the fish, we recorded latitude and longitude using a Garmin GPSMAP 64s (Garmin Ltd., Olathe, Kansas, USA). We also measured water temperature [ºC], dissolved oxygen [mg L^{-1}], and conductivity [µS cm^{-1}] using a Hach Rugger DO/pH/Conductivity Field Kit (Hach, Loveland, Colorado, USA). Measurements were taken at the point of fish collection, but only once per sampling site.

We downloaded daily weather information (maximum, minimum and mean daily temperature and rainfall) for each sampling site from the ECA&D database (http://www.ecad.eu), at a resolution of 0.1 degrees latitude/longitude. For our analyses, we used averaged values across 120 days (the day of sampling plus the preceding 119 days), in order to account for seasonal and yearly weather variation (Table B.1). We further measured distance from the sea [m] for each population using Google Maps (http://www.google.com/maps).

We condensed weather data and population-specific environmental parameters via principal component analysis (PCA) with Varimax rotation. Using Varimax rotation, the axes of the multidimensional space (i.e., the orginal environmental variables) are rotated in order to maximise the sum of the variance of the squared loadings (i.e., the correlation between the PCs and the original variable), resulting in increased loadings for a lower number of variables, thus facilitating the interpretation of the results (Kaiser 1958). We obtained 3 PCs with eigenvalues > 1.0 that accounted for 74.28% of the overall variation (Table B.2); these were used as explanatory environmental variables in all subsequent analyses (hereafter named EPC1-3).

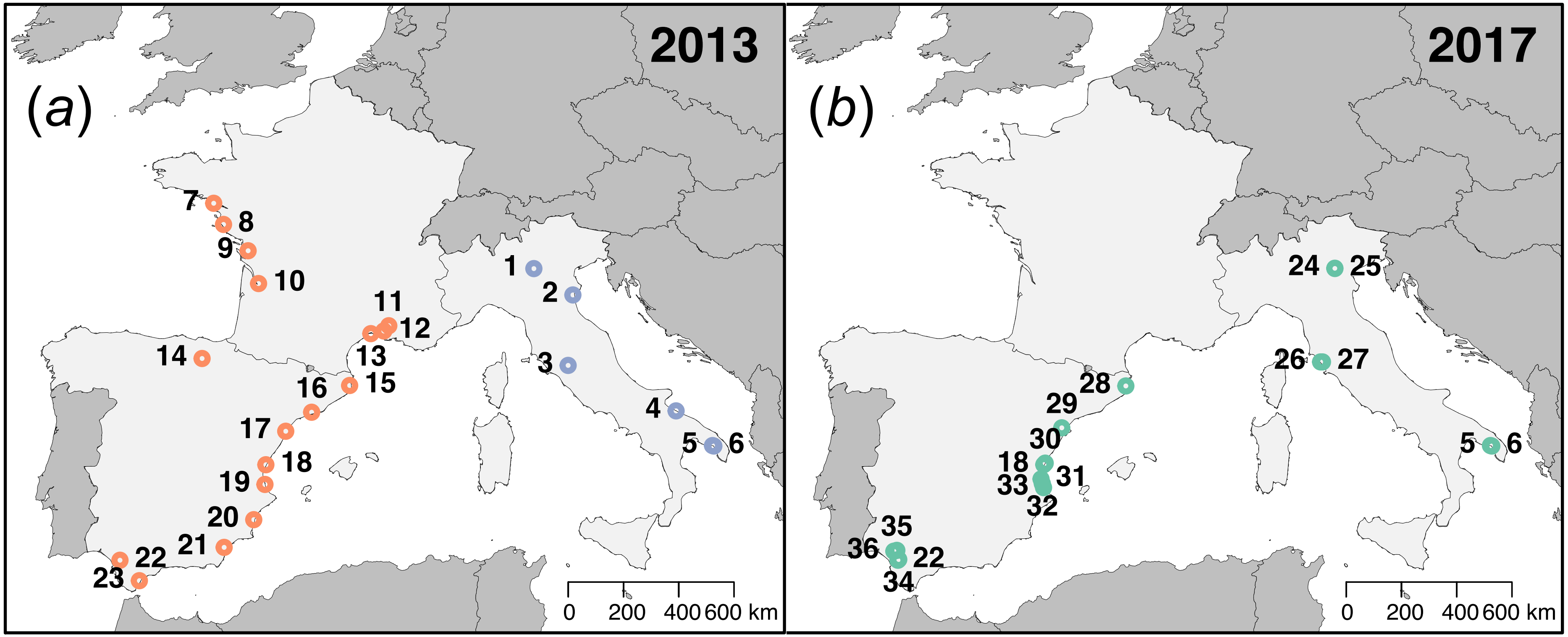

Figure 3.1. Location of sampling sites in Italy, France and Spain. (*a*) Sites sampled in 2013. Blue points represent sites sampled in May 2013, orange points sites sampled in September 2013. (*b*) Sites sampled in July/August of 2017 (green).

Population genetic analyses

We amplified nuclear microsatellites and conducted population genetic analyses to determine the genetic structure among 23 *G. holbrooki* populations in Europe. We extracted DNA from *N* = 660 ethanol-preserved tissue samples using the NucleoSpin Tissue Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's recommendations. We used primer pairs established for *G. affinis* (Spencer et al. 1999, Purcell et al. 2011) and *G. holbrooki* (Zane et al. 1999), which were arranged in three separate multiplex reactions (reaction 1: Gaaf10, Gaaf11, Gaaf13, Gafμ3; reaction 2: Gaaf7, Gaaf9, Gaaf15, Gaaf16, Gaaf22, Gafμ2, Gafμ6; reaction 3: Gafμ1, Gafμ4, Gafμ7, Mf-13) and amplified using the Type-it Microsatellite PCR kit (Qiagen, Hilden, Germany) under thermocycling conditions as follows: initial denaturation for 5:00 min at 95°C, 30 cycles of 1:30 min at 60°C, and 0:30 min at 72°C, followed by a final extension step for 30:00 min at 60°C. Each 5 μL reaction mix included 2.5 μL Type-it master mix, 0.4 μL primer mix, 0.4 μL Q-solution, 0.9 μL RNAse-free water, and 0.8 μL template DNA. Fragment sizes were scored manually after electrophoresis on a Beckman Coulter capillary sequencer CEQ 2000, using an internal size standard (Beckman Coulter, Brea, CA, USA).

Body-shape analysis

We analysed body-shape variation using geometric morphometrics (Rohlf and Marcus 1993, Zelditch et al. 2012) on 1,331 wild-caught individuals (620 sexually mature males and 711 pregnant females). In the laboratory, we took standardised photographs of the left body side of each individual using a

Canon EOS 400D DSLR camera with a 50 mm macro lens (Canon Inc., Tokyo, Japan) mounted on a copy stand. Photos were collated using tpsUtil32 Version 1.70 (Rohlf 2016a), and one of us (F.S.) added 15 landmarks to each photo using tpsDig232 Version 2.26 (Rohlf 2016b): (1) tip of the upper lip, (2) posterior end of the head, (3) anterior and (4) posterior insertion of the dorsal fin, (5) top, (6) middle and (7) bottom of the caudal peduncle, (8) posterior and (9) anterior insertion of the anal fin, (10) where the ventral end of the operculum meets the body, (11) anterior margin of the eye orbit, (12) centre of the eye, (13) posterior margin of the eye orbit, (14) dorsal and (15) ventral insertion of the pectoral fin, following Jourdan et al. (2016). We corrected for bending (which happened to some individuals during preservation) by using the "unbend specimen" function in tpsUtil32; the program uses quadratic regression to correct the bending effects. To that end, two temporary landmarks were added along the lateral line of the fish, and were subsequently removed again (Ouyang et al. 2018).

Using tpsRelW32 Version 2.26 (Rohlf 2016c), we performed a relative warps analysis (Zelditch et al. 2012) on wild-caught fish, from which we obtained 3 relative warps (RWs) that described 91.2% of the total body-shape variation. Visual representation using thin-plate splines showed that RW1 mainly described differences between males and females, while RW2 and RW3 mainly described differences in body depth and head size (Fig. B.3). These RWs were used as shape variables for all subsequent analyses on wild-caught fish (Table B.5). Centroid size (the sum of the quadratic distances of each landmark from their centroid) was used as a covariate to control for body-size effects.

We conducted a second relative warps analysis on 145 (65 males and 80 females) wild-caught and 116 (59 males and 57 females) laboratory-reared (F_2) individuals from four populations (see section on common-garden rearing below) and again retained 3 RWs that described 92.8% of the body-shape variation. Similar to our previous analysis, RW1 mainly described differences due to sexual dimorphism, RW2 mainly described differences in head size, and RW3 differences in body and caudal-peduncle depth (Fig. B.7). We used these RWs in subsequent analyses to address the (broad-sense) heritability of population variation in body shape.

Life-history analysis

Following well established life-history protocols (Reznick and Endler 1982, Riesch et al. 2016), we dissected the fish in order to quantify the following traits: male and female standard length (SL [mm]), dry weight [mg], lean weight [mg] (dry weight after fat extraction) and fat content [% of dry weight], male gonadosomatic index (GSI [%]; testis dry weight divided by the sum of somatic and testis dry weight), female fecundity (number of developing embryos) and reproductive allocation (RA [%]; total offspring dry weight divided by the sum of maternal somatic and total offspring dry weight), offspring dry weight [mg], offspring lean weight [mg], and offspring fat content [%] (Table B.6, B.7). We further assessed the developmental stage of each embryo following Riesch et al. (2011), with embryonic stages ranging from 2 (fertilized oocyte with blastodisc present) to 50 (embryo ready to be born).

In order to meet statistical assumptions of normality of residuals, we $\log_{10}$-transformed (SL, adult lean weight and offspring lean weight), square root-transformed (fecundity), or arcsine(square root)-transformed (GSI, RA, adult and embryo fat content) all life-history variables. We subsequently *z*-transformed all variables to obtain unit-free variables with equal variance for all subsequent analyses.

Common-garden rearing

To examine whether phenotypic divergence between populations is the result of phenotypic plasticity or based on evolved (genetic) differences, we also evaluated fish from a population-level common garden-rearing experiment. Laboratory stocks were available from four populations sampled in 2013, three from Italy (Torre Castiglione, Comacchio and Lago di Garda), and one from Spain (Zadorra; see Table B.1 for details). All stocks were founded by dozens of individuals each in May 2013 (Italy) and August 2013 (Spain), and maintained as randomly outbred populations in 200-L tanks in the temperature-controlled Animal Facility of the University of Frankfurt (two tanks per population). All stocks were exposed to identical environmental conditions (i.e., 12:12 h light:dark cycle, constant 24ºC water temperature). Fish were fed twice daily with commercial flake food (TetraMin® Tetra GmbH), frozen chironomid larvae, bosmids and *Artemia salina* shrimps. In order to separate different generations and to avoid cannibalistic behavior, we introduced single pregnant females into a net cage (20 × 35 × 30 cm; 5 mm mesh size), placed in the upper portion of individual 60-L tanks. Neonates were collected daily and transferred to new 200-L tanks. Random samples of mature males and pregnant females of the second laboratory generation (F_2) were collected in December 2014.

Statistical analyses

Unless stated otherwise, statistical analyses were conducted using IBM® SPSS® Statistics for Macintosh v.21 (2012), and IBM® SPSS® Statistics for Windows v.22 (2013; IBM Corp., Armonk, NY).

Population genetic structure

We reanalysed and included microsatellite data from a previous US American sampling (see Riesch et al. 2018). We used the software STRUCTURE 2.3.4104 to calculate individual assignment probabilities (*Q*-values) to varying numbers of genetically distinct clusters (*K*). For each value of *K* = 1–25, ten iterations were run using the admixture model with a burn-in period of 20,000 generations, followed by a sampling phase of 50,000 iterations. We detected the uppermost level of population differentiation with the method presented by Evanno et al. (2005) using the web-based tool STRUCTURE HARVESTER 0.6.93105. Moreover, we calculated genetic distances between populations (Nei's D_A; Nei et al. 1983) using Populations 1.2.32. Based on the distance matrix, we constructed a neighbour-joining tree in MEGA X (Kumar et al. 2018) to infer phylogeographic relationships among native (North American) and invasive (European) populations. Results from additional population genetic analyses are presented in Appendix B2.

Phenotypic variation among populations (wild-caught specimens)

We initially screened our data for differences in life history and body shape between populations, genetic clusters, and years. After confirming significant population differences (Table B.8), we proceeded to investigate the effects of climate and environmental parameters (EPC1-3) on phenotypic variation.

We analysed body-size variation using an ANCOVA with SL as the dependent variable, 'year' and 'sex' as factors, as well as 'EPC1-3' as covariates. We further analysed the influence of environmental parameters on phenotypic differentiation by running three separate multivariate general linear models (GLMs) on body shape, and male and female life histories, respectively. In the GLM on body shape, we used RW1-3 as dependent variables, 'sex' and 'year' as factors, and 'EPC1-3' and 'centroid size' as covariates. In the GLM on male life-history traits, we used 'year' as factor, and 'EPC1-3' as well as 'SL' as covariates. We used a similar model structure to analyse female life histories, but added 'embryonic stage of development' as an additional covariate. In all analyses, we first included all two-way interactions but removed terms with $P > 0.1$ from the final models. For multivariate models, we approximated F-ratios using Wilks' Lambda and estimated effect sizes using partial η^2. In the main article, we focus on the most important effects of climate-dependent and -independent phenotypic diversity, and we report all other effects in Appendix B3, B4.

Comparison of wild-caught vs. laboratory-raised specimens

We analysed variation in body shape using MANCOVA with 'sex', 'population' (four levels), and 'generation' (two levels: wild-caught vs. F_2

laboratory-raised) as factors, and centroid size as a covariate. In this and in subsequent models, significant effects of ‘population’ would indicate that some degree of population-level phenotypic differences was retained in laboratory-reared individuals.

For life-history traits, we first analysed variation in SL using an ANOVA with ‘sex’, ‘population’, and ‘generation’ as factors. We then ran two sex-specific MANCOVAs on the remaining life-history traits, coding ‘population’ and ‘generation’ as factors. In the model on male life histories, SL served as a covariate, while in the model on female life histories, SL and ‘embryonic stage of development’ served as covariates. Again, the initial models included all possible interactions between factors and covariates, but interactions were removed from the final models if $P > 0.1$.

To obtain a more direct measure of the repeatability of population differences (i.e., broad sense heritability), we calculated intraclass correlation coefficients (ICCs) for all RWs and life-history traits separately using estimated marginal means from the abovementioned models (i.e., correcting for the influence of centroid size, SL and/or embryonic stage of development; Riesch et al. 2013, Eifert et al. 2015). Usually, ICC-values will range from 0 to 1, with values below 0.50 suggesting poor repeatability, 0.50-0.75 moderate repeatability, 0.75-0.90 good repeatability, and values above 0.90 suggesting excellent repeatability (e.g., Koo and Li 2016).

Results

Population genetic structure

We successfully genotyped $N = 660$ individuals and detected $K = 2$ as the uppermost hierarchical level of population structure according to Evanno et al. (2005; Appendix B2). We found that the two major genetic clusters in the STRUCTURE analysis correspond to North American (blue) and European populations, respectively (green; Fig. 3.2*b*). The second highest ΔK was found for $K = 4$, followed by $K = 15$ (Fig. B.2). STRUCTURE runs for $K = 4$ revealed population genetic structure within European populations, dividing them into an Italian/French (yellow) and a Spanish cluster (brown; with the exception of the northernmost French population, Brière; site number 7; Fig. 3.2*b*). A phylogenetic tree based on Nei's genetic distances suggested a common origin of all European mosquitofish, whereby the population from North Carolina (NC) appears to be ancestral to European mosquitofish (Fig. 3.2*a*). Furthermore, it confirmed two clusters among European populations, one comprising French/Italian and one comprising Spanish *G. holbrooki* (and a single French population). In general, allelic richness (A) was significantly reduced in European populations compared to populations in their native range ($t_{31} = 6.614$, $P < 0.001$; Fig. B.1*a*).

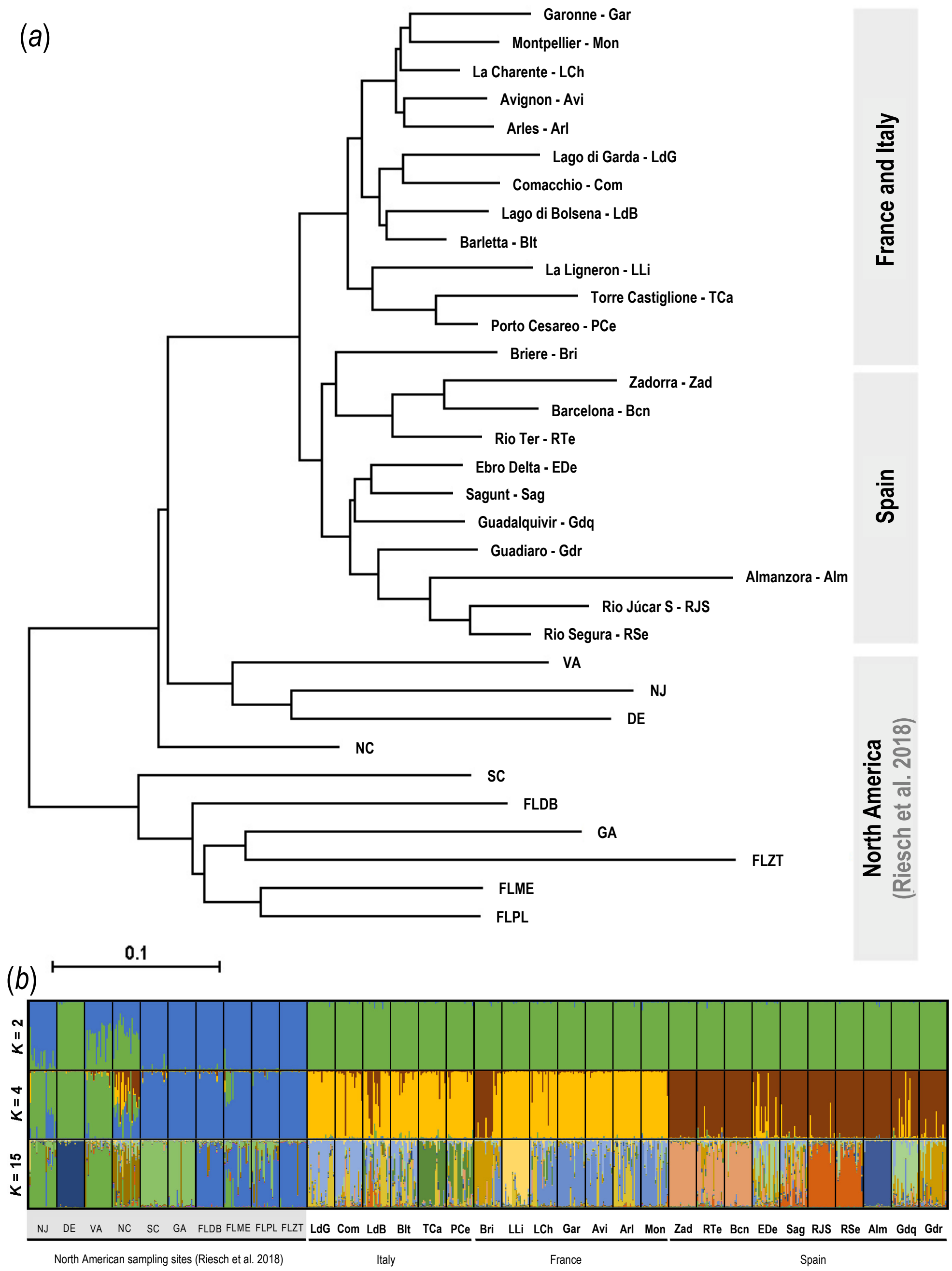

Figure 3.2. (*a*) Neighbour-joining tree, using Nei's genetic distances, based on fragment length polymorphisms of 15 nuclear microsatellites in native (North American) and invasive (European) populations. (*b*) Results from STRUCTURE v.2.3.4104. *K* = 2 was the most likely number of genetically distinct clusters according to the method provided by Evanno et al. (2005), followed by *K* = 4 and *K* = 15 (Fig. B.2). Each individual is represented by a vertical bar, which is partitioned into *K*-coloured segments representing its estimated likelihood of membership (*Q*) to each of the identified clusters. Figure created by J. Jourdan.

Phenotypic differences among populations

In our preliminary analyses (Appendix B3; Table B.8), we found significant differences between populations and genetic clusters in body shape and male and female life-history traits. The population-effect in particular was the second most important source of phenotypic diversity, after ‘sex’ (for body shape; $\eta_p^2 = 0.175$) and ‘SL’ for both male and female life histories ($\eta_p^2 = 0.220$ and 0.265, respectively).

Sexual dimorphism in wild-caught mosquitofish

Sexual dimorphism (factor ‘sex’) was the main source of phenotypic variation in both, the ANCOVA on SL and the MANCOVA on body shape ($\eta_p^2 = 0.688$ and 0.962, respectively; Appendix B3). Females were bigger than males (Tables B.6, B.7), and were characterised by enlarged abdominal regions, while in males the anal fin (modified into the gonopodium) was shifted anteriorly (Fig. B.3).

Climate-dependent phenotypic variation

We found significant effects of climate (‘EPC1’-effect; Table B.2) on body size, body shape, and male and female life histories. However—based on η_p^2—the importance of these effects was relatively minor (see Appendix B3 for a detailed breakdown of these effects) and several climatic responses changed between sampling years.

In colder climates (i.e., northern and Italian populations) males had rounder and deeper bodies compared to fish from warmer climates (i.e., southern and Spanish populations), while females showed the opposite pattern, being characterised by deeper bodies in southern populations (‘sex × EPC1’-effect; Fig. 3.3*a*; Tables 3.1*a*, 3.2). Moreover, fish had increased body size in colder/northern populations than in warmer/southern ones (‘EPC1’-effect), but this response was stronger in males than in females (‘sex × EPC1’-effect; Fig. 3.3*b*).

When considering life-history traits, ‘EPC1’ had significant effects in males on both lean weight and GSI, as males from northern populations had increased lean weight and GSI (Fig. 3.3*c*; Tables 3.1*b*, 3.3). In colder regions females had higher reproductive investment (RA), while fat content increased in warmer regions, but only in bigger females and not in smaller ones (‘SL × EPC1’-effect). Some effects varied, however, between sampling years, as females were characterised by increased RA in populations from colder regions in 2017, but this effect disappeared in 2013 (‘year × EPC1’-effect; Fig. 3.3*d*; Tables 3.1*c*, 3.4).

Climate-independent phenotypic variation

Dissolved oxygen (DO), distance from the sea (both ‘EPC2’) and conductivity (‘EPC3’, Table B.2) had high axis loadings in the PCA on environmental variation. In high-oxygen, close-to-the-sea environments, fish were smaller, and females had bigger offspring than in low-oxygen, far-from-the-sea ones. Similarly to climate-effects (see above), responses to dissolved oxygen and distance to the sea tended to vary between sampling years, as the negative effect of DO on fecundity, and its positive effect on embryo weight and fat content were present in 2013 and all but disappeared in 2017 (Fig. 3.3*e*).

In habitats characterised by high conductivity, fish had deeper bodies, males were heavier, and females had reduced investment into reproduction than in low-conductivity environments. Furthermore, increased conductivity had a negative effect on male, female, and embryo fat contents (Fig. 3.3*f*). Again, in 2017 fish had more streamlined bodies, higher fecundity and RA, but lower embryo fat in high-conductivity environments, while the pattern was reversed in 2013.

Table 3.1. MANCOVAs investigating the effects of environmental principal components (EPC1-3; Table B.2) on phenotypic variation of invasive mosquitofish. (*a*) Male and female body shape; (*b*) male life-history traits and (*c*) female life-history traits. Significant effects are highlighted in bold.

	Factor	*F*	Degrees of freedom	*P*	Partial η^2
(*a*) Male and female body shape	**Sex**	11,097.447	3, 1318	**< 0.001**	0.962
	Centroid size	13.252	3, 1318	**< 0.001**	0.029
	Year	21.908	3, 1318	**< 0.001**	0.047
	EPC1	6.465	3, 1318	**< 0.001**	0.015
	EPC2	1.616	3, 1318	0.184	0.004
	EPC3	9.957	3, 1318	**< 0.001**	0.022
	Sex × EPC1	3.437	3, 1318	**0.016**	0.008
	Sex × EPC3	2.125	3, 1318	0.095	0.005
	Year × EPC1	10.337	3, 1318	**< 0.001**	0.023
	Year × EPC3	8.324	3, 1318	**< 0.001**	0.019
(*b*) Male life-history traits	**SL**	1,048.004	3, 610	**< 0.001**	0.838
	Year	70.811	3, 610	**< 0.001**	0.258
	EPC1	21.536	3, 610	**< 0.001**	0.096
	EPC2	1.149	3, 610	0.329	0.006
	EPC3	5.057	3, 610	**0.002**	0.024
	SL × EPC3	2.142	3, 610	0.094	0.010
	Year × EPC1	2.754	3, 610	**0.042**	0.013
(*c*) Female life-history traits	**SL**	1,725.397	6, 693	**< 0.001**	0.937
	Embryo stage	19.386	6, 693	**< 0.001**	0.144
	Year	16.597	6, 693	**< 0.001**	0.126
	EPC1	11.674	6, 693	**< 0.001**	0.092
	EPC2	6.211	6, 693	**< 0.001**	0.051
	EPC3	18.416	6, 693	**< 0.001**	0.138
	SL× EPC1	5.541	6, 693	**< 0.001**	0.046
	SL × EPC2	9.297	6, 693	**< 0.001**	0.074
	SL × EPC3	8.725	6, 693	**< 0.001**	0.070
	Year × EPC1	6.205	6, 693	**< 0.001**	0.051
	Year × EPC2	7.593	6, 693	**< 0.001**	0.062
	Year × EPC3	17.469	6, 693	**< 0.001**	0.131

Table 3.2. *Post-hoc* ANCOVAs on body-shape variation. Alpha-levels were corrected for multiple comparisons, such that $\alpha' = 0.017$. Significant effects are highlighted in bold.

Dependent variable	Factor	*F*	Degrees of freedom	*P*	Partial η^2
RW1	**Sex**	285,444.920	1, 1320	**< 0.001**	0.956
	Centroid size	10.837	1, 1320	**0.001**	0.008
	Year	34.036	1, 1320	**< 0.001**	0.025
	EPC1	10.746	1, 1320	**0.001**	0.008
	EPC2	1.174	1, 1320	0.279	0.001
	EPC3	10.800	1, 1320	**0.001**	0.008
	Sex × EPC1	0.046	1, 1320	0.831	< 0.001
	Sex × EPC3	0.432	1, 1320	0.511	< 0.001
	Year × EPC1	18.598	1, 1320	**< 0.001**	0.014
	Year × EPC3	12.870	1, 1320	**< 0.001**	0.010
RW2	**Sex**	14.104	1, 1320	**< 0.001**	0.011
	Centroid size	23.295	1, 1320	**< 0.001**	0.017
	Year	0.563	1, 1320	0.453	< 0.001
	EPC1	0.474	1, 1320	0.491	< 0.001
	EPC2	4.713	1, 1320	0.030	0.04
	EPC3	6.523	1, 1320	**0.011**	0.005
	Sex × EPC1	0.261	1, 1320	0.609	< 0.001
	Sex × EPC3	4.437	1, 1320	0.035	0.003
	Year × EPC1	10.867	1, 1320	**0.001**	0.008
	Year × EPC3	1.144	1, 1320	0.285	0.001
RW3	**Sex**	6.463	1, 1320	**0.011**	0.005
	Centroid size	16.210	1, 1320	**< 0.001**	0.012
	Year	30.736	1, 1320	**< 0.001**	0.023
	EPC1	6.975	1, 1320	**0.008**	0.005
	EPC2	< 0.001	1, 1320	0.987	< 0.001
	EPC3	5.654	1, 1320	0.018	0.004
	Sex × EPC1	10.048	1, 1320	**0.002**	0.008
	Sex × EPC3	2.103	1, 1320	0.147	0.002
	Year × EPC1	11.949	1, 1320	**0.001**	0.009
	Year × EPC3	15.268	1, 1320	**< 0.001**	0.011

Table 3.3. *Post-hoc* ANCOVAs on male life-history traits. Alpha-levels were corrected for multiple comparisons, with α' = 0.017. Significant effects are highlighted in bold.

Dependent variable	Factor	*F*	Degrees of freedom	*P*	Partial η^2
Lean weight	**SL**	2,969.739	1, 612	**< 0.001**	0.829
	Year	4.616	1, 612	0.032	0.007
	EPC1	32.096	1, 612	**< 0.001**	0.050
	EPC2	0.618	1, 612	0.432	0.001
	EPC3	5.680	1, 612	**0.017**	0.009
	SL × EPC3	0.012	1, 612	0.915	< 0.001
	Year × EPC3	5.549	1, 612	0.019	0.009
Fat content	**SL**	5.874	1, 612	**0.016**	0.010
	Year	22.716	1, 612	**< 0.001**	0.036
	EPC1	0.073	1, 612	0.321	< 0.001
	EPC2	1.334	1, 612	0.248	0.002
	EPC3	8.061	1, 612	**0.005**	0.013
	SL × EPC3	0.068	1, 612	0.794	< 0.001
	Year × EPC3	0.355	1, 612	0.551	0.001
GSI	**SL**	13.938	1, 612	**< 0.001**	0.022
	Year	182.258	1, 612	**< 0.001**	0.229
	EPC1	41.060	1, 612	**< 0.001**	0.063
	EPC2	1.454	1, 612	0.228	0.002
	EPC3	4.155	1, 612	0.042	0.007
	SL × EPC3	6.355	1, 612	**0.012**	0.010
	Year × EPC3	3.812	1, 612	0.051	0.006

Table 3.4. *Post-hoc* ANCOVAs on female life-history traits. Alpha-levels were corrected for multiple comparisons, with α' = 0.008. Significant effects are highlighted in bold.

Dependent variable	Factor	*F*	Degrees of freedom	*P*	Partial η^2
Lean weight	**SL**	9,964.501	1, 698	**< 0.001**	0.935
	Embryo stage	1.007	1, 698	0.316	0.001
	Year	25.932	1, 698	**< 0.001**	0.036
	EPC1	5.130	1, 698	0.024	0.007
	EPC2	4.025	1, 698	0.045	0.006
	EPC3	2.369	1, 698	0.124	0.003
	SL × EPC1	6.964	1, 698	0.009	0.010
	SL × EPC2	0.285	1, 698	0.594	< 0.001
	SL × EPC3	11.663	1, 698	**0.001**	0.016
	Year × EPC1	1.203	1, 698	0.273	0.002
	Year × EPC2	3.252	1, 698	0.072	0.005
	Year × EPC3	6.328	1, 698	0.012	0.009
Fat content	SL	0.005	1, 698	0.942	< 0.001
	Embryo stage	0.002	1, 698	0.967	< 0.001
	Year	22.793	1, 698	**< 0.001**	0.032
	EPC1	0.284	1, 698	0.594	< 0.001
	EPC2	1.163	1, 698	0.281	0.002
	EPC3	9.421	1, 698	**0.002**	0.013
	SL × EPC1	7.550	1, 698	**0.006**	0.011
	SL × EPC2	6.736	1, 698	0.010	0.010
	SL × EPC3	5.619	1, 698	0.018	0.008
	Year × EPC1	0.036	1, 698	0.850	< 0.001
	Year × EPC2	5.108	1, 698	0.024	0.007
	Year × EPC3	0.629	1, 698	0.428	0.001

Table 3.4 continued.

Dependent variable	Factor	*F*	Degrees of freedom	*P*	Partial η^2
Fecundity	**SL**	497.898	1, 698	**< 0.001**	0.416
	Embryo stage	7.918	1, 698	**0.005**	0.011
	Year	4.785	1, 698	0.029	0.007
	EPC1	5.120	1, 698	0.024	0.007
	EPC2	3.647	1, 698	0.057	0.005
	EPC3	3.611	1, 698	0.058	0.005
	SL × EPC1	0.030	1, 698	0.864	< 0.001
	SL × EPC2	0.031	1, 698	0.860	< 0.001
	SL × EPC3	36.905	1, 698	**< 0.001**	0.050
	Year × EPC1	0.345	1, 698	0.557	< 0.001
	Year × EPC2	12.383	1, 698	**< 0.001**	0.017
	Year × EPC3	37.582	1, 698	**< 0.001**	0.051
Embryo fat content	SL	5.021	1, 698	0.025	0.007
	Embryo stage	87.785	1, 698	**< 0.001**	0.112
	Year	21.293	1, 698	**< 0.001**	0.030
	EPC1	0.179	1, 698	0.672	< 0.001
	EPC2	0.297	1, 698	0.530	0.001
	EPC3	35.073	1, 698	**< 0.001**	0.048
	SL × EPC1	0.118	1, 698	0.731	< 0.001
	SL × EPC2	0.436	1, 698	0.509	0.001
	SL × EPC3	0.842	1, 698	0.359	0.001
	Year × EPC1	1.665	1, 698	0.197	0.002
	Year × EPC2	28.538	1, 698	**< 0.001**	0.039
	Year × EPC3	13.467	1, 698	**< 0.001**	0.019
Embryo weight	**SL**	29.268	1, 698	**< 0.001**	0.040
	Embryo stage	19.377	1, 698	**< 0.001**	0.027
	Year	1.040	1, 698	0.308	0.001
	EPC1	1.139	1, 698	0.286	0.002
	EPC2	17.184	1, 698	**< 0.001**	0.024
	EPC3	5.701	1, 698	0.017	0.008
	SL × EPC1	4.145	1, 698	0.042	0.006
	SL × EPC2	0.551	1, 698	0.458	0.001
	SL × EPC3	18.787	1, 698	**< 0.001**	0.026
	Year × EPC1	1.018	1, 698	0.313	0.001
	Year × EPC2	9.364	1, 698	**0.002**	0.013
	Year × EPC3	1.620	1, 698	0.204	0.002
RA	**SL**	7.969	1, 698	**0.005**	0.011
	Embryo stage	0.753	1, 698	0.386	0.001
	Year	11.194	1, 698	**0.001**	0.016
	EPC1	14.490	1, 698	**< 0.001**	0.020
	EPC2	0.108	1, 698	0.743	< 0.001
	EPC3	29.410	1, 698	**< 0.001**	0.040
	SL × EPC1	0.250	1, 698	0.617	< 0.001
	SL × EPC2	3.099	1, 698	0.079	0.004
	SL × EPC3	11.134	1, 698	**0.001**	0.016
	Year × EPC1	12.529	1, 698	**< 0.001**	0.018
	Year × EPC2	0.774	1, 698	0.379	0.001
	Year × EPC3	54.441	1, 698	**< 0.001**	0.072

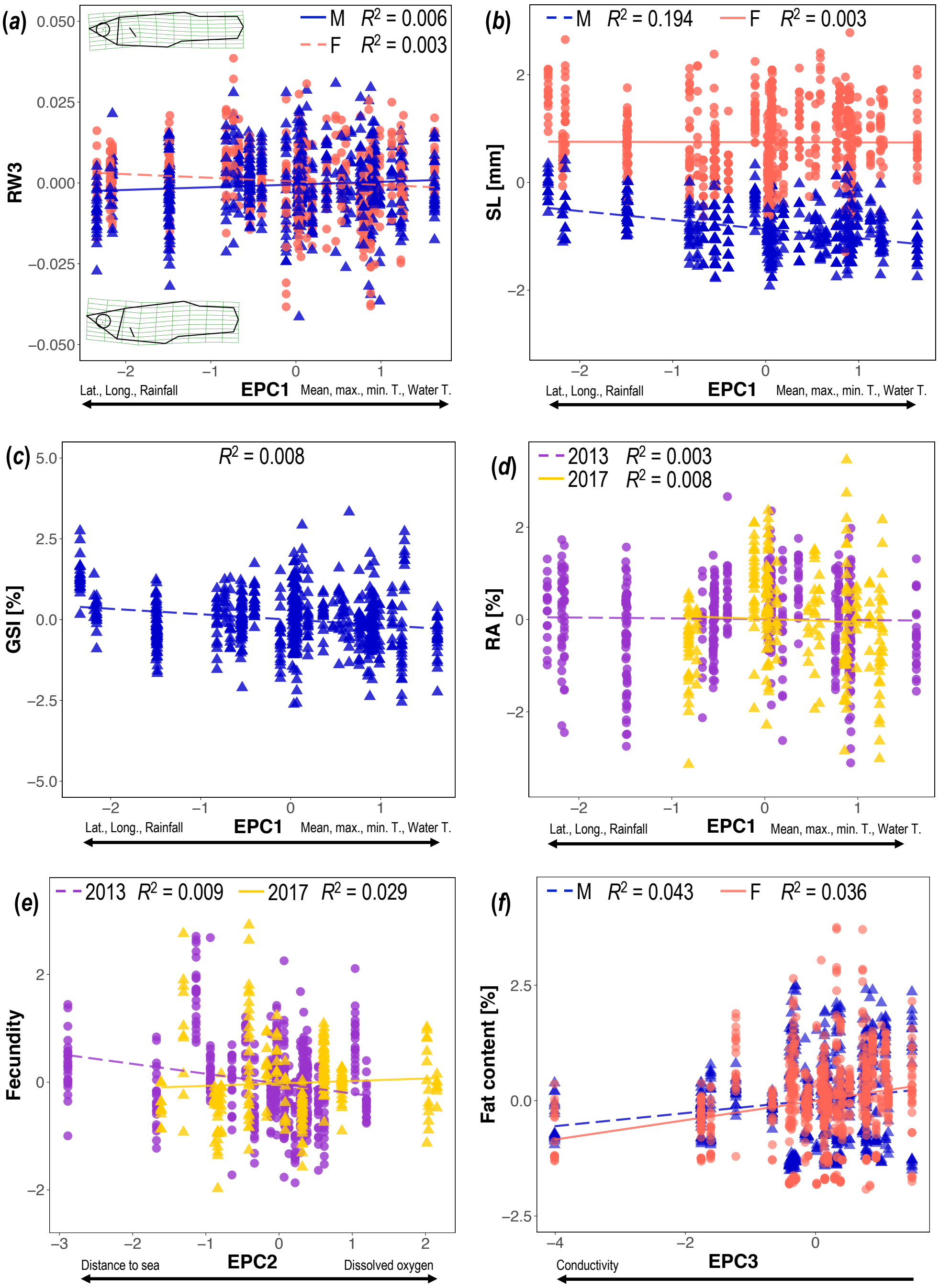

Figure 3.3. Partial regression plots of climate-dependent (EPC1) and -independent (EPC2, EPC3) variation of body shape and life histories in wild-caught *G. holbrooki*. The plots (and regression lines) are derived from ANCOVAs and data points represent residuals corrected for all the other terms in the models. (*a*) Body-shape (RW3) variation; (*b*) body-size (SL) variation along EPC1. Blue triangles: males, red circles: females. (*c*) variation of male GSI along EPC1; (*d*) variation of female reproductive allocation (RA) along EPC1 in fish sampled in 2013 (purple circles) and 2017 (yellow triangles); (*e*) fecundity variation along EPC2 in females sampled in 2013 and 2017; (*f*) fat content variation along EPC3 in males (blue triangles) and females (red circles).

Heritability of phenotypic differentiation

The comparison between wild-caught and laboratory-reared *G. holbrooki* revealed—among others—significant effects of 'population', 'generation', and 'population × generation' on body size, shape, and life histories (see Appendix B4 for a detailed breakdown of these effects). Both males and females modified their phenotypes in the laboratory, but most of these modifications were population-specific ('population × generation'-effect). Laboratory-raised fish were bigger (estimated marginal mean ± s.e.m.: 30.23 ± 0.32 mm) than wild-caught individuals (25.25 ± 0.26 mm), while wild-caught fish had slightly deeper bodies, shorter caudal peduncles, smaller eyes, and their anal fin was positioned more posteriorly compared to laboratory-reared individuals. In the laboratory, males were characterised by higher fat content and lower lean weight and GSI (Table B.10), while females had higher fat content, lower fecundity, and produced offspring with greater lean weight and fat content (Table B.13).

Among population differences detected in wild-caught individuals, we did not find significant broad sense heritability for any life.history and body-shape trait. While male SL (ICC = 0.628, 95% CI: -0.686, 0.956; Fig. 3.4*a*), male lean weight (ICC = 0.556, 95% CI: -0.794, 0.929; Fig. 3.4*b*), and embryo fat content (ICC = 0.734, 95% CI: -0.712, 0.951; Fig. 3.4*c*) appeared to have moderate broad sense heritability, 95% confidence intervals bounded zero in all cases. All other traits exhibited poor repeatability (fecundity: ICC = 0.407, $P = 0.338$; ICC < 0.200 in all other cases).

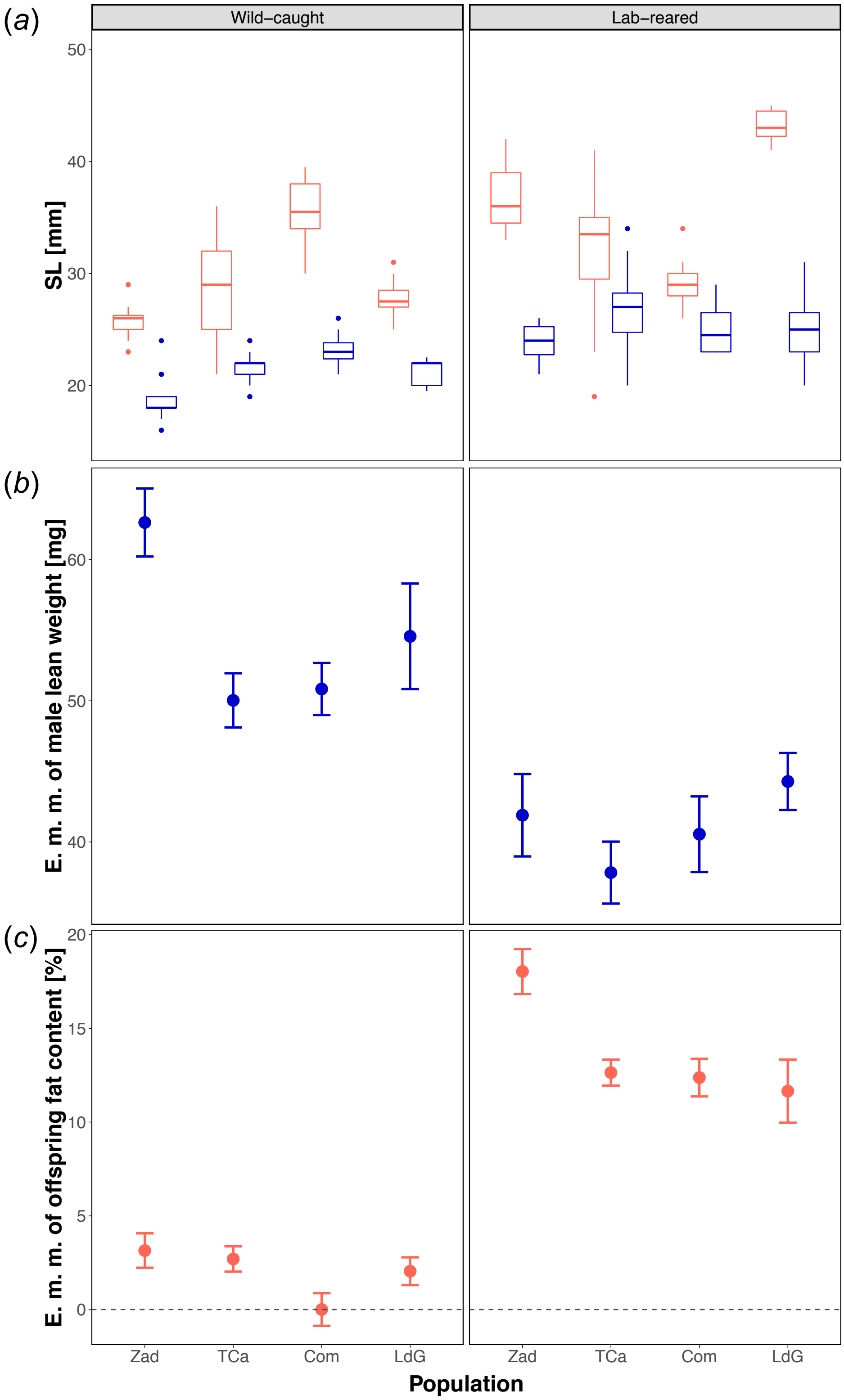

Figure 3.4. Visualisation of significant population-by-generation interactions in wild-caught and lab-reared mosquitofish. (*a*) Box plot of male and female body size (SL). (*b*) Estimated marginal means (e. m. m.) ± SE of male lean weight (derived from MANCOVA and estimated for SL = 23.17 mm). (*c*) Estimated marginal means ± SE and of embryo fat content (derived from MANCOVA and estimated for SL = 30.78 mm and embryonic stage of development = 15.38). Zad: Zadorra; TCa: Torre Castiglione; Com: Comacchio; LdG: Lago di Garda.

Discussion

We found pronounced phenotypic variation in our wild-caught mosquitofish (for which population genetic analyses confirmed a single European introduction). Phenotypic variation was driven by both climate-dependent and -independent environmental parameters, however several phenotypic responses differed between sampling years, suggesting a strong role of phenotypic plasticity in driving these patterns. This interpretation was largely confirmed by our common garden experiment, where most traits received low broad-sense heritability estimates.

Population genetic structure

Historical records identify Spain as the first point of introduction of *G. holbrooki* from North Carolina in 1920; from there, mosquitofish were further introduced to Italy and France in 1921, and to southern Russia in 1924 (Grapputo et al. 2006, Vidal et al. 2010). While it is impossible to exclude additional introductions, as the use of mosquitofish as mosquito-control agents remained common practice throughout the 20th century and still occurs today (Ghosh and Dash 2007, Sarwar 2015; but see Azevedo-Santos et al. 2017), our population genetic analyses support the hypothesis of a single introduction from one source population (see also Vidal et al. 2010). Specifically, populations from the central US East Coast appear to be the closest sister populations to all invasive mosquitofish populations in Europe (matching the reported North Carolina origin). The common ancestry amongst invasive populations suggests that phenotypic variation might be driven by selective pressures across the European invasive range, and does not simply reflect multiple introductions of populations with independent evolutionary histories from within the species' native distribution range.

Climate-dependent and -independent phenotypic variation

Mosquitofish responded to the climatic (mainly latitudinal) gradient across Europe by growing to a larger body size in colder environments (i.e., both in northern Italy and northern France), and this response was stronger in males than females. Additionally, males from colder environments had deeper, rounder bodies with relatively smaller heads, while this response was opposite in females. The increase in body size in colder climates matches patterns previously described for both native *G. holbrooki* in the USA (Riesch et al. 2018), and invasive *G. affinis* from China (Ouyang et al. 2018). Increased body size in colder populations is common among endotherms, where it is linked to decreased heat loss (Bergmann's Rule; Bergmann 1847, Gaston et al. 2008), but has also been described for several ectotherms (Otalla-Tarraga and Rodrigues 2007, Vinarski 2014, Osorio-Canadas et al. 2016), including fish (Belk and Houston 2002, Knouft, 2004). In mosquitofish, winter mortality can reach 85-99% in northern populations (Sloterdijk et al. 2015, Cheng et al. 2018), and bigger body size likely leads to increased overwinter survival (Riesch et al. 2018)—an effect that was also reported for other fishes (e.g., bluegill sunfish, *Lepomis macrochirus*, Cargnelli and Gross 1996; sailfin mollies, *Poecilia latipinna*, Trexler et al. 1992). While a previous study did not find evidence for sex-biased overwinter mortality (Cheng et al. 2018), males are likely to be more vulnerable than females (Geiser 1924, Krumholz 1948), which might explain the greater increase in body size in males than females towards the north.

The climatic responses in fat content and body shape did not align with what has been reported for the species' native range (Riesch et al. 2018). One would expect higher fat content to be favoured at low temperatures, since a previous study has identified body fat reserves as a key component of overwinter survival (Reznick and Braun 1987). Mosquitofish usually have higher fat reserves in populations subject to harsher winters (Meffe and Snelson 1993, Ouyang et al. 2018, Riesch et al. 2018), and similar patterns were reported for other fishes (e.g. silverside, *Menidia menidia*, Schultz and Conover 1997). However, climate had no effect on male fat reserves, and big females actually increased their fat content in southern populations. One possible explanation is that these climatic responses were overshadowed by the negative effects of conductivity on both male and female fat content (see below), and conductivity did not follow a latitudinal gradient (Table B.1). Alternatively, seasonal effects might be invoked as a confounding factor for the lack of responses in fat content in northern populations, as mosquitofish increase their fat reserves towards the end of the reproductive season (Meffe and Snelson 1993, Reznick et al. 2006) and use those energy reserves during winter (Reznick and Braun 1987), while some of our fish (Italian samples from 2013) were sampled at the beginning of the reproductive season.

Body shape responses in females aligned with what has been described for native *G. holbrooki* (Riesch et al. 2018), but were opposite to those observed in invasive *G. affinis* (Ouyang et al. 2018), whereas in males these patterns were reversed. While it could be expected that rounder and deeper bodies are favoured in colder climates—and males' responses aligned with this prediction—both native and invasive female *G. holbrooki* from northern populations actually had shallower bodies (Riesch et al. 2018; this study). At present, we lack a convincing explanation for those conflicting patterns. However, body-shape variation is tightly linked to differences in life-history traits (Wesner et al. 2011), such as body fat reserves. In invasive *G. affinis*, for example, individuals from northern populations were characterised by increased body fat content and rounder, deeper bodies, while females of invasive *G. holbrooki* did not share these responses. Also, body-shape variation in livebearing fishes is not only influenced by temperature, but also by other environmental parameters such as predation (Langerhans 2009) and flow regime (Langerhans and Reznick 2009), which we could not assess in our current study. Future studies will need to explore potential (co-)variation of those selection factors along climate gradients.

Among climate-independent environmental variables, dissolved oxygen, distance to the sea (both EPC2), and conductivity (EPC3) affected mosquitofish phenotypes. Along EPC2, sampling sites were characterised by either being high-oxygen, close-to-the-sea, or low-oxygen, inland environments. Even though all but two sites had O_2-concentrations greater than what is usually considered hypoxic (i.e., < 2 mg L^{-1}; Chapman 2015; Table B.1), EPC2-variation affected male and female life histories. In high-oxygen, close-to-the-sea environments, mosquitofish exhibited reduced body size and produced bigger offspring. Moreover, females had reduced fecundity in high-oxygen environments in 2013, while this pattern was reversed in 2017. The few existing studies on the phenotypic effects of relatively small differences in DO suggest that indirect effects might drive these responses. For instance, the negative correlation between oxygen levels and fecundity in *G. holbrooki* from rice fields in Portugal (Cabral and Marques 1999) may be linked to high-oxygen levels translating into greater prey availability and population densities (Cabral et al. 1998), both of which favour the production of bigger offspring and indirectly result in a lower fecundity. Lower fecundity under elevated DO was also found in Brazilian *Phalloptychus januarius* (Santi et al. 2020), where piscivorous predators are highly tolerant to hypoxia, and so selection from predation may drive the observed pattern in that system.

Lastly, differences in conductivity—which we used as a proxy for salinity (Poisson 1980)—affected both body shape and life-history traits. Fish were characterised by deeper bodies and reduced fat content, and females reduced their reproductive allocation in high-conductivity environments; yet again, most of these responses varied between fish sampled in 2013 and 2017. A previous study on invasive *G. holbrooki* also showed a negative effect of salinity on both body condition and reproduction (Alcaraz and García-Berthou 2007). Even though *G. holbrooki* is known to tolerate salinities higher than sea water (Chervinski 1983), and all sampling sites included here had fresh to brackish water (the sole exception being a site close to Montpellier, Mon; Table B.1), our results confirm conductivity (and salinity), as a potential limiting factor regarding the range expansion of mosquitofish (Alcaraz and García-Berthou 2007, Alcaraz et al. 2008). Similar negative effects of salinity on reproduction have also been found in other livebearing fish species (e.g., *Poecilia velifera*, Neves et al. 2019).

Heritability of phenotypic variation

All life-history and body-shape traits showed highly plastic responses, as our analyses highlighted the lack of significant repeatability for all life-history and body-shape traits. This led to different phenotypes in the laboratory-raised populations compared to those quantified for natural populations. These phenotypic responses to the laboratory environment—rounder, deeper bodies, increased body size and fat content, coupled with reduced GSI in males and increased offspring size and reduced fecundity in females—appear to follow prediction from life-history theory in case of stable environments with high population densities (*K*-selection; Reznick et al. 2002). Moreover, they also likely reflect increased food availability (i.e., *ad libitum* feeding) compared to food availability in natural populations (Stearns 1989, Reznick et al. 2002, Spoljaric and Reimchen 2007).

Plasticity is usually the first organismal response to changes in selection regimes (Ghalambor et al. 2007), allowing otherwise suboptimal genotypes to shift their phenotypes in an adaptive direction (Hendry 2015). As a result of this, phenotypic plasticity can “shield” genotypes from selection and reduce the speed of genetic differentiation (Hendry 2015). This is true especially in the case of adaptive phenotypic plasticity, or plasticity that produces phenotypes that are favoured by selection in a given environment (Ghalambor et al. 2007). Indeed, when plastic phenotypes are sufficiently close to the fitness optimum in different environments, populations might not undergo genetic evolution at all (Ghalambor et al. 2007). In mosquitofish populations, body-shape and life-history differences are not only driven by (somewhat predictable) climatic differences, but also by other (possibly unpredictable) environmental factors, like population density or food availability (Riesch et al. 2018). We argue that unpredictable environmental variation favours fast-acting adaptive plasticity, which in turn reduces the opportunity for local adaptation in most traits (Reed et al. 2010).

It has been suggested that plasticity in traits important to fitness—in the presence of environmental variation similar to that experienced in the native range—would favour invasiveness and the colonization of new environments (Hendry 2015), and support for this idea comes from an array of invasive species (Trussell and Smith 2000, Rosecchi et al. 2001, Duncan et al. 2003, Dzialowski et al. 2003, Chown et al. 2007, Ghalambor et al. 2007). Higher levels of plasticity in invasive species, compared to their non-invasive counterparts, have been reported for both plants (Davison et al. 2011) and animals (Dingemanse et al. 2010; but see Bossdorf et al. 2005, Richards et al. 2006). In this context, our current study provides further support for this argument.

Conclusions

Our study demonstrates, for the first time, that phenotypic plasticity drives most of the observed phenotypic diversity. This highlights the importance of phenotypic plasticity during the colonization of new environments, and how phenotypic plasticity might hinder (or, in certain cases, facilitate) rapid genetic change. This has important implications for our understanding of species' invasiveness. We call for future studies using comparative transcriptomics to understand how environmental variation affects the expression of developmental genes and their regulatory elements, as well as comparative genomics to understand what regions of the genome have already undergone evolutionary divergence.

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Chapter 4: Geographical and temporal variation of multiple paternity in invasive mosquitofish (*Gambusia holbrooki*, *Gambusia affinis*).

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Abstract

Multiple paternity (MP) increases offspring's genetic variability, which could be linked to invasive species' evolvability in novel distribution ranges. Shifts in MP can be adaptive, with greater MP in harsher/colder environments or towards the end of the reproductive season, but climate could also affect MP indirectly via its effect on reproductive life histories. We tested these hypotheses by genotyping $N = 2{,}903$ offspring from $N = 306$ broods of two closely related livebearing fishes, *Gambusia holbrooki* and *Gambusia affinis*. We sampled pregnant females across latitudinal gradients in their invasive ranges in Europe and China, and found more sires per brood and a greater reproductive skew towards northern sampling sites. Moreover, examining monthly sampling from two *G. affinis* populations, we found MP rates to vary across the reproductive season in a northern Chinese, but not in a southern Chinese population. While our results confirm an increase of MP in harsher/more unpredictable environments, path analysis indicated that, in both cases, the effects of climate are likely to be indirect, mediated by altered life histories. In both species, which rank amongst the 100 most invasive species worldwide, higher MP at the northern edge of their distribution likely increases their invasive potential and favours range expansions, especially in light of the predicted temperature increases due to global climate changes.

Key-words

Global climate change, Evolvability, Europe, China, *Gambusia*, Invasion potential.

Introduction

Invasive species are a global threat to biodiversity as well as ecosystem health and stability (Mooney and Cleland 2001, Simberloff et al. 2013, Gallardo et al. 2016, Ricciardi et al. 2017). They are one of the leading causes of extinction worldwide (Clavero and García-Berthou 2005), and aquatic ecosystems are especially vulnerable (Gallardo et al. 2016). While fishes rank amongst the most threatened groups of aquatic animals (Gozlan et al. 2010) they are also amongst the most commonly introduced organisms, with 624 species reported to have become invasive due to fish farming, ornamental fish trade, and fisheries (Gozlan 2008). Some species are more successful invaders than others, but our understanding of which traits increase invasion success is still limited (Catford et al. 2019). Besides *r*-selected life-history traits (Sakai et al. 2001), the ability to increase genetic diversity amongst offspring via multiple paternity (MP) has been suggested to explain invasion success of both invertebrate and vertebrate species (e.g., Miller et al. 2010, Yue et al. 2010).

MP can occur as a consequence of both male and female reproductive behaviour, such as male sexual harassment (Pizzari and Birkhead 2000) and sexual coercion (Kelly et al. 1999, Bisazza et al. 2001), or active female solicitation of multiple mating (Jennions and Petrie 2000). In the latter case, females may gain direct and indirect benefits from multiple copulations (Jennions and Petrie 2000). For instance, by mating multiply, otherwise sperm-limited females can obtain sufficient sperm (Borowsky and Kallman 1976). Alternatively, multiple mating can limit the negative effects of suboptimal mate choice (Hamilton 1990) or directly increase the genetic quality of females' offspring (Jennions and Petrie 2000, Pitcher et al. 2003), resulting in an increased offspring fitness (e.g., guppies, *Poecilia reticulata*, Evans and Magurran 2001, Ojanguren et al. 2005). Lastly, females can mate multiply in order to increase the genetic variation of their offspring as a genetic bet-hedging strategy (Yasui 2001). In this way females increase the likelihood that at least some of their offspring will be viable in unpredictable and/or unfavourable environments (Zane et al. 1999, Mäkinen et al. 2007, Simons 2011, García-González et al. 2014).

Several studies have shown that levels of MP are influenced—directly or indirectly—by multiple biotic and abiotic environmental factors. Depending on the mechanism underlying MP, levels of MP might either in- or decrease under adverse environmental conditions. For example, under high predation conditions, guppy males increase their rates of coercive mating (Godin 1995) thereby increasing both the proportion of multiply-sired broods and the number of sires per brood (Kelly et al. 1999, Neff et al. 2008). Climate also influences MP, for instance in yellow-bellied marmots (*Marmota flaviventris*), where the proportion of multiply sired broods decreases in years with more snow coverage, simply because this decreases the chance for males and females to meet (Martin et al. 2014). Similarly, MP in sand lizards (*Lacerta agilis*) is lower in colder years (Olsson and Madsen 2001, Olsson et al. 2011), and frequencies of extra pair paternity in birds increase in habitats characterized by high climate variability and low predictability (Botero and Rubenstein 2012).

Here, we analysed how climate influences geographical and temporal variation of MP in two highly invasive species of mosquitofish: *Gambusia* *holbrooki* and *Gambusia affinis* (Poeciliidae). Both species are livebearers with internal fertilisation that are native to North America, and they are similar in their appearance, biology and general ecology (Pyke 2005). Since the 1920s, they have been widely introduced as mosquito control agents (Benejam et al. 2009), with rare-to-non-existent effects on mosquitoes but mostly negative effects on other native species (Kottelat and Whitten 1996). As a result, both species are considered to rank among the 100 most harmful invasive species worldwide (Lowe et al. 2000). Today, invasive *G. holbrooki* occur across Southern Europe, Australia, Northern and parts of Central Africa, whereas *G. affinis* is established across Asia, including mainland China (Welcomme 1992).

Mosquitofish are characterised by highly seasonal reproductive behaviour: the reproductive season usually starts in mid-spring and lasts until mid-autumn (Pyke 2005), and water temperature appears to be the most important driver of the reproductive cycle (Fraile et al. 1994). In both, *G. affinis* and *G. holbrooki*, females mate multiply and MP is commonly found in most broods (Zane et al. 1999). Unlike several other poeciliids, male *Gambusia* usually lack brightly coloured nuptial ornaments (Magurran 2005; but see Martin et al. 2014) and typically do not exhibit courtship, but rely on forced copulations, so-called “gonopodial thrusts” (Bisazza et al. 2001). Nevertheless, females have been shown to prefer bigger over smaller males (Chen et al. 2018) and groups of males over single males (Bisazza et al. 2001), suggesting that they may be able to bias paternity of their monthly broods.

We assessed levels of MP in European *G. holbrooki* and Chinese *G. affinis* across a latitudinal gradient. We further quantified monthly MP variation across the reproductive season in two Chinese populations, a southern and a northern one. We specifically tested two different hypotheses. We predicted that MP levels would be higher in harsher, more unpredictable environments (hypothesis 1), i.e., in northern populations of our geographical sampling, and towards the end of the reproductive season in our temporal sampling (Haynes and Cashner 1995, Karlsson et al. 2005, Reznick et al. 2006). In both cases, higher offspring quality (Møller and Alatalo 1999, Evans and Magurran 2001, Pitcher et al. 2003, Pilastro et al. 2007) and/or genetic diversity (Grapputo et al. 2006) should be favoured in more unfavourable environmental conditions (Botero and Rubenstein 2012). In harsher environments, in fact, higher-quality offspring are expected to have a survival advantage over lower-quality ones, being more able to resist starvation and/or environmental extremes (Sogard 1997). Alternatively, higher offspring genetic diversity can protect females against complete reproductive failure in unpredictable environments (bet-hedging, Yasui 2001).

Nevertheless, multiple, potentially interacting and not always mutually-exclusive mechanisms have been shown to influence MP in livebearing fishes, and climate might therefore not have a direct influence on MP. A brief summary of these mechanisms—including mechanisms supporting our specific *a priori* hypotheses and additional explanations for variation in MP—are provided in Table 4.1. For example, number of sires per clutch usually increases as fecundity increases (Neff et al. 2008), and fecundity has been shown to increase towards northern populations in native *G. holbrooki* from the East Coast of the USA (Riesch et al. 2018). At the same time, increased male body size in northern populations could lead to a reduction of MP in those populations, as bigger males are able to monopolise access to females (Bisazza and Marin 1991), and smaller males have higher efficiency in coercive copulation attempts due to their higher manoeuvrability (Pilastro et al. 1993). An additional level of complexity is introduced by potential geographic variation in demographic factors like adult sex ratios that may, for instance, affect the efficiency of alternative male mating strategies (Table 4.1 for an overview). Thus, population differences in MP could be due to spurious effects, indirectly mediated by climate through altered life-history traits or altered population demography (hypothesis 2). If indirect climate effects (via altered life histories and/or population demography) play a role here, several different scenarios are possible, including population-level and monthly differences in MP that do not always change predictably with climate or seasonal temperature patterns, respectively. It could even be possible that life histories or demographic factors have a stronger total influence on variation in MP than climate variation *per se*.

Table 4.1. Summary of mechanisms that have been suggested to explain—directly or indirectly—MP variation. Our summary focusses on livebearing fishes (family Poeciliidae) and on our study species *Gambusia affinis* and *G. holbrooki* in particular. Sires: numbers of sires per brood; skew: reproductive skew (unevenness in the paternity contribution of different males to a given brood). Geo: predicted effects on the geographical variation of MP; Temp: predicted effects on temporal variation of MP (hypothesis 1); LH: predicted direct effects of altered life-history traits on MP variation (hypothesis 2).

Selective/ inducing agent	**Trait**	**Effect**	**References**
Male sexual coercion	Sires	▲ at increased population densities and male-biased sex ratios. Densities decrease in northern/colder populations. Geo.: ▼ in northern and/or colder populations. Temp.: ▲ towards the end of the reproductive season (months with higher population densities).	Head et al. 2017
	Skew	▼ at increased population densities and male-biased sex ratios. Geo.: ▲ in northern and/or colder populations. Temp.: ▼ towards the end of the reproductive season (months with higher population densities).	Emlen and Oring 1977
(Cryptic) female choice	Sires	▲ female choice in harsher, more unpredictable environments: females “trade up” and mate with increasingly attractive males. Geo.: ▲ in northern and/or colder populations. Temp.: ▲ towards the end of the reproductive season (colder months).	Stearns 1983 Møller and Alatalo 1999 Evans and Magurran 2001 Pitcher et al. 2003
	Skew	Paternity biased towards the most attractive male(s) via cryptic female choice. Geo.: ▲ in northern and /or colder populations. Temp.: ▲ towards the end of the reproductive season (colder months).	Pilastro et al. 2004 Pilastro et al. 2007 Botero and Rubenstein 2012
Female choice (bet hedging)	Sires	▲ genetic variation of females’ offspring as an “insurance” against non-viable phenotypes in unpredictable environments. Geo.: ▲ in northern and/or colder populations. Temp.: ▲ towards the end of the reproductive season.	Yasui 2001 Grapputo et al. 2006 Mäkinen et al. 2007 Simons 2011
	Skew	Paternity evenly distributed across all sires in order to maximize offspring genetic variation. Geo: ▼ in northern and/or colder populations. Temp.: ▼ towards the end of the reproductive season.	García-González et al.2014

Table 4.1 continued.

Male-male competition	Sires & Skew	Bigger males are able to monopolize access to females. LH: ▼ with increased SL (standard length). Geo.: ▼ in northern and/or colder populations (where males achieve bigger body size). Temp.: ▼ towards the end of the reproductive season (when males achieve bigger body size).	Bisazza and Marin 1991 Riesch et al. 2018
Male insemination efficiency	Sires & Skew	Smaller males have higher insemination efficiency in coercive copulations, as they are more manoeuvrable and more difficult for the females to spot. Geo.: ▼ (sires), ▲ (skew) in northern and/or colder populations (where males are bigger). Temp.: ▼ (sires), ▲ (skew) towards the end of the reproductive season (when males are bigger).	Pilastro et al. 1997 Pilastro et al. 2003
Female fecundity (sampling error)	Sires	The ability to find multiply sired offspring in a randomly selected subset of embryos increases with fecundity. LH: ▲ in females with higher fecundity.	Neff et al. 2008
Female fecundity	Sires	Previous studies have highlighted that the increase in number of sires with fecundity cannot be explained solely by sampling errors. LH: ▲ in females with higher fecundity.	Neff et al. 2008
Other life-history effects	Sires & Skew	Mosquitofish life histories are affected by latitude-dependent and -independent environmental variation. Male and female body size, reproductive investment and female fecundity further vary across the reproductive season. LH: strong influence of life histories on MP.	Abney and Rakocinski 2004 Ouyang et al. 2018 Riesch et al. 2018
Sex ratio	Sires	▲ under male-biased sex ratio. Temp.: ▲ towards the end of the reproductive season (when the sex ratio becomes male-biased).	Zulian et al. 1995 Head et al. 2017
Inbreeding	Sires	Inbred males have reduced multiple paternity shares, due to selection against inbred males. In guppies, females cryptically prefer unrelated males (mediated by the ovarian fluid).	Gasparini and Pilastro 2011 Marsh et al. 2017 Vega-Trejo et al. 2017
Predation	Sires & Skew	High predation: reduced female choice, increase of male coercive sexual behaviour, which increases MP. Geo.: ▲ (sires), ▼ (skew) in high-predation populations Temp.: ▲ (sires), ▼ (skew) in summer months, when predation is higher (e.g., by *Diplonychus esakii* on Chinese *G. affinis*).	Neff et al. 2008 Ouyang et al. 2017
Overwinter mortality	Sires	Lower overwinter mortality in bigger females, which have higher fecundity and attract a higher number of males. Geo.: ▲ in northern/colder populations.	Haynes 1993 Reznick et al. 2006
Number of sires	Skew	Positive correlation between sires and skew, as the number of sires per brood limits the upper value of reproductive skew.	theoretical consideration

Materials and methods

Ethical statement

The current study does not include experiments involving live animals. All experimental procedures were approved by the Animal Welfare commissioner at the Department of Animal Science of the College of Animal Science and Technology, Northwest A&F University and by the College Named Animal Care and Welfare Officer of the School of Biological Sciences, Royal Holloway University of London (authorisation number: 503-2017-05-13-14-53-PCBA015). All procedures were performed in accordance with the relevant guidelines of China and the European Union (Directive 2010/63/EU). Sampling permits were obtained from the Chinese Government (Standards for the investigation of reservoir fishery resources SL 167-2014), and from the relevant European local authorities (permit numbers: Determinazione N°: 530, Decreto N°: 8169, Decreto N°: 7478, Expedient EPI 55/2017, Ref. SGYB/AF).

Sample collection and climatic data

Geographic sampling of *G. holbrooki* and *G. affinis*

We collected *Gambusia holbrooki* at 10 sites in Spain and Italy between July and August 2017 (Fig. 4.1*a* and Table C.1*a*), across an 8.5° latitudinal gradient, and *G. affinis* at 11 sites in mainland China between April 2017 and April 2018 (Fig. 4.1*b* and Table C.1*b*) across a 17.3° latitudinal gradient, in order to assess geographic variation in MP. In addition, we repeatedly sampled two Chinese populations, namely C2 – Ankang (May – Oct. 2016) and C11 – Beihai (Apr. – Sept. 2016; Table C.1*c*) once per month during the reproductive season in order to further investigate how patterns of MP vary across the year. For our monthly sampling, we were able to additionally assess population densities using catch-per-unit-effort (CPUE; numbers of fish caught per one hour of catching; Richards and Schnute 1992) and adult sex ratios (ASR; number of sexually-mature males divided by the number of mature females caught). All collection sites were stagnant or slow-flowing water bodies with dense riparian vegetation. Fish were captured using dip nets (2 mm mesh size), immediately euthanized with an overdose of clove oil, and then preserved in 96% ethanol for subsequent analyses.

We downloaded climatic data for our study sites (1970–2000) from WorldClim Version 2 (http://worldclim.org/version2) at 30 arc-seconds resolution (Fick and Hijmans 2017). We extracted the following site-specific means: (1) annual temperature, (2) maximum temperature of the warmest month, (3) minimum temperature of the coldest month, (4) annual temperature difference (by subtracting the minimum monthly temperature from the maximum monthly temperature), and (5) annual precipitation. We obtained population-specific information on (6) altitude and (7) distance to the sea from Google Earth (http://earth.google.com/; Table C.2; see Ouyang et al. 2018). We condensed all seven variables via principal component analysis (PCA) with Varimax rotation and retained three environmental principal components (EPCs) that described over 84% of the variance (Table C.3*a*).

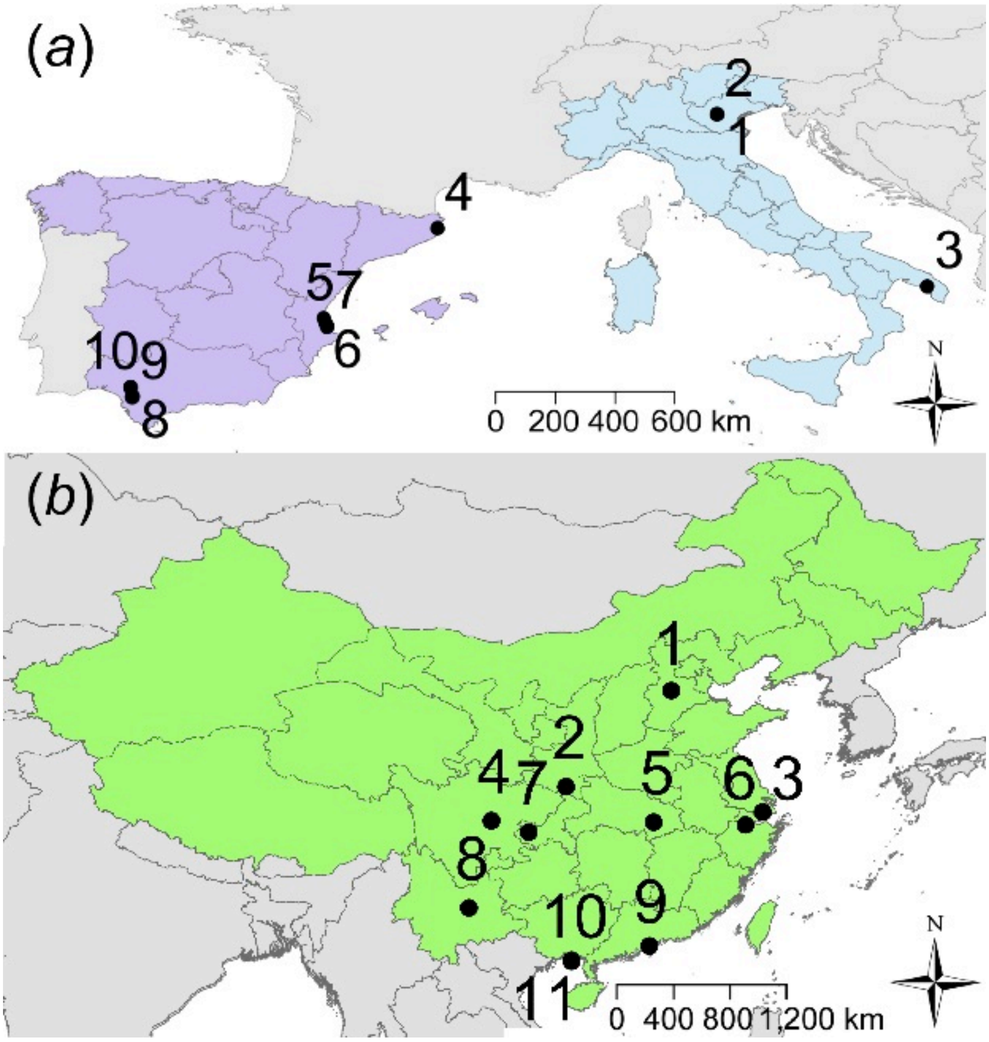

Figure 4.1. Sampling sites of (*a*) invasive *G. holbrooki* in Europe and (*b*) invasive *G. affinis* in mainland China (see Table C.1 for details). The map was generated using ArcMap v 10.3, and created by J. Gao.

Monthly sampling in Ankang and Beihai

For Ankang and Beihai, we downloaded monthly temperature data (mean maximum and minimum temperatures, the maximum temperature of the warmest day and minimum temperature of the coldest day; http://www.tianqihoubao.com/lishi/) for the reproductive season of 2016. We then condensed these data using PCA and extracted one temperature PC that described over 77% of the variance (Table C.3*b*-*d*). This PC, population density (CPUE) and adult sex ration (ASR), were used as covariates in subsequent analyses.

Assessing MP

In the laboratory, we measured the standard length (SL) of each female to the nearest 0.01 millimetre using a digital calliper. We then opened the abdominal cavity and extracted all embryos (if present). We separated the embryos using forceps, determined their developmental stage following Riesch et al. (2011), and we quantified fecundity as the number of developing embryos for each female. Only females harbouring embryos developed enough for the DNA extractions to work reliably (stage 20 or higher) were considered in the assessment of MP. We quantified multiple paternity on a maximum of 10 individuals per sampling site (or monthly sampling), depending on the number of females meeting this criterion, with a minimum of 5 females for the Porto Cesareo population (see Table C.5 for details). For each female, MP was quantified on a subset of 10 randomly chosen embryos if fecundity was higher than 10, otherwise all embryos were used.

Microsatellite analysis

We extracted genomic DNA from entire embryos and from females' pectoral fin tissue using the Universal Genomic DNA Kit (CWBIO, Beijing, China). We genotyped females and their corresponding offspring using eight polymorphic nuclear microsatellite loci (multiplex PCR_1: Gaaf22, Gafμ2, Gafμ3, Gafμ4; multiplex PCR_2: Gaaf7, Gaaf10, Gaaf13, Gafμ7; Table C.6; Purcell et al. 2011, Spencer et al. 1999). Each multiplex PCR was performed in a total volume of 20 µL using 10 µL 2×Taq MasterMix (CWBIO, Beijing, China), 0.3–0.5 µL forward and reverse primers (10 µM) and 80–500 ng genomic template DNA. Thermocycling was conducted under the following cycling conditions: initial denaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 30 s (denaturation), 60°C for 30 s (primer annealing), 72°C for 30 s (elongation), and a final elongation step at 72°C for 10 min. We scored fragment sizes using GeneMapper version 4.0 (Applied Biosystems, Foster City, CA) after electrophoresis on an ABI3730 sequencer, using Liz500 as the internal size standard.

Quantifying patterns of MP

We calculated the polymorphic information content (PIC; Botstein et al. 1980) and combined exclusion probabilities (Marshall et al. 1998) for each population/month using CERVUS 3.0.7 (Kalinowski et al. 2007); descriptive statistics can be found in Appendix C. We estimated numbers of sires per clutch using COLONY version 2.0.6.4 (Wang 2004, Jones and Wang 2010). COLONY reconstructs sibship and estimates numbers of putative sires and parental genotypes, based on a likelihood method applied to multi-locus genotype data. Prior to paternity estimation, we checked data for nulls—caused by scoring error, null alleles, or mutation—by counting allelic mismatches between offspring and mothers (Jones et al. 2010, Girndt et al. 2012). The highest mismatching frequency detected was 0.05 in our Beihai population at locus Gafu4. We computed the reproductive skew among sires based on our COLONY results as described in Neff et al. (2008). Skew was summarised by first calculating the effective number of sires as $1/\Sigma(r_{si}$/brood size)^2; where r_{si} is the number of offspring assigned to sire i, and the summation is over all sires contributing to a brood. We then expressed the reproductive skew as 1 – (effective number of sires/actual number of sires) (Neff et al. 2008). In order to meet statistical assumptions of normality of residuals, we square root-transformed numbers of sires and fecundity, and arcsine(square root)-transformed reproductive skew. We subsequently *z*-transformed all variables to obtain unit-free variables with equal variance for all subsequent analyses.

Assessment of life-history traits

We obtained population/monthly means of life-history traits for both our geographical and temporal samples using well-established protocols (Riesch et al. 2015, 2018, Ouyang et al. 2018). For the geographical sample, we used a subset of 19 populations (leaving out Baoding and Shenzhen for lack of mature/pregnant individuals). We thus dissected 132 male and 146 female *G. holbrooki* (5–29 individuals per site), as well as 196 male and 176 female *G. affinis* (9–30 individuals per site). For the temporal sample, we dissected 167 males and 123 females from Ankang (16–30 individuals per month), as well as 158 males and 145 females from Beihai (19–30 individuals per month; Table C.7). Please note that these fish were collected at the same time as the females that we used to quantify MP (see above), but are different individuals. Therefore, they provided us with a different (population-level) measure of fecundity.

We measured standard length (SL [mm]), lean weight [mg] and adult fat content [%] for males and females. We further determined male gonadosomatic index (GSI [%]; testis dry weight divided by the sum of testis and somatic dry weight) as well as female fecundity, embryo lean weight [mg], embryo fat content [%] and reproductive allocation (RA [%]; the dry weight of the developing embryos divided by the sum of the embryo and female somatic dry weight). In order to fulfil model assumptions of normality of residuals and to avoid scaling effects, we $\log_{10}$-transformed SL, lean weight and embryo lean weight, square-root transformed fecundity, and arcsine (square root) transformed fat content, RA and GSI, after which we *z*-transformed all variables.

Statistical analyses

Unless stated otherwise, all statistical analyses were performed using IBM SPSS Statistics 22.0 (IBM Inc., Chicago, IL, USA).

Geographic sampling of *G. holbrooki* and *G. affinis*

We investigated the potential involvement of climate in driving population differences in MP in our geographic sampling (confirmed by preliminary GLMs, see Table C.8) by running separate general linear models (GLMs) for number of sires and reproductive skew. In both models, we used ‘species’ as a fixed factor, and the three environmental PCs and fecundity (in this case the measure of fecundity derived from the females used to assess MP) as covariates. Previous studies have shown that both female fecundity (Neff et al. 2008, Zeng et al. 2017) and SL (Herdman et al. 2004, Deaton 2008) influence patterns of MP in poeciliid fishes. In our analyses we only included fecundity, given the strong correlation between fecundity and SL (Pearson’s correlations; *G. holbrooki*: $r_P = 0.682$, $P < 0.001$; *G. affinis*: $r_P = 0.730$, $P < 0.001$). We initially included all two-way interaction terms between the fixed factor and covariates, but eliminated interaction term(s) from the final analysis if $P > 0.1$. We evaluated the relative importance of each term in the final model using partial eta squared (η_P^2).

Given the relatively low explanatory power of environmental PCs in the GLMs (Table 4.2), we employed maximum likelihood path analysis to test the relative importance of direct effects of environmental factors on MP variation and of indirect effects mediated by life-history variation. For this, we used a subset of the total data for which population-level life-history data were available (all $N = 10$ *G. holbrooki* populations and $N = 9$ *G. affinis* populations; see above). First, we screened population-level life-history traits for potential allometric effects (i.e., significant correlations with 'SL') or dependency on 'embryonic stage of development' by using linear regressions. Each life-history trait was therefore regressed against 'SL' and 'embryonic stage of development' (analyses not shown). In case of significant results, we used the residuals from the models for subsequent analyses. We thus corrected male and female lean weight as well as (population-level) fecundity for SL, and embryo lean weight, embryo fat content and RA for 'embryonic stage of development'. Afterwards, we calculated site-specific means for each life-history trait, which were then condensed via a PCA. We thus obtained four life-history PCs that described 82.51% of the variation in the original life-history traits (Table C.4*a*). We then ran path analysis using IBM SPSS Amos 21 (IBM Inc., Chicago, IL, USA). We used 'species' and our three original environmental PCs (see above) as exogenous variables, and our newly derived four life-history PCs as well as our two measures of MP (residuals of number of sires and reproductive skew derived from a linear regression were each measure of MP was corrected for individual female fecundity) as endogenous variables. The model selection was performed as follows: we first constructed the full model (i.e., paths from each higher-level node to each lower-level node). Then, in a stepwise process, we deleted one by one the paths with the smallest path coefficients (and $P > 0.15$) until we arrived at a model with all path coefficients having $P \leq 0.05$. In each step, we used Akaike's Information Criterion corrected for small sample size, (AIC*c*; Burnham and Anderson 2002), in order to evaluate model fit. In other words, the resulting final model comprised the most "important" paths. Each time, we checked the model for multicollinearity (average VIF < 3.7 in all cases), and we calculated and tested for significance all direct and all total path coefficients (which reflect standardised partial regression coefficients) using 2,000 bootstrap resampling iterations.

Monthly sampling in Ankang and Beihai

We asked whether differences in MP across the reproductive season in the Ankang and Beihai populations (confirmed in preliminary GLMs; Table C.8) can be ascribed, at least in part, to climatic variation. To this end, we ran GLMs on numbers of sires and reproductive skew using 'population' as a fixed factor, and our temperature PC as well as fecundity (measured from the same females used to assess MP) as covariates.

Again, temporal patterns of MP were further evaluated via path analysis. Similarly to the analysis of geographical variation of MP, we conducted PCA on population-specific means for life-history traits (if needed, corrected for SL and 'embryonic stage of development'). This resulted in three life-history PCs (Table C.4*b*). This time, we included in the model the temperature PC, CPUE and ASR as exogenous variables, and our newly derived three life-history PCs as well as both measures of MP (corrected for female fecundity) as endogenous variables. Model selection then followed the procedure described above.

Results

Occurrence of MP in invasive mosquitofish

For summary statistics on the microsatellite markers (e.g., polymorphic information contents and exclusion probabilities for the second parent), please refer to Appendix C. We found a high percentage of multiply sired broods in each of the tested populations (see Appendix C, Table C.9 for details).

Geographical variation in MP

GLM results on effects of climate and individual variation in female fecundity

Both numbers of sires and reproductive skew were significantly affected by 'species', fecundity and environmental PC1 (see Table 4.2 for GLM results and Table C.4 for descriptive statistics). Moreover, numbers of sires were significantly affected by the interaction terms 'species × environmental PC1' and 'species × fecundity' (Table 4.2*a*).

Congruent with our predictions, northern populations of both species had a higher number of sires per brood than southern ones (environmental PC1 effect; Fig. 4.2*a*; Fig. C.1). Moreover, numbers of sires generally increased with increasing fecundity (Fig. 4.2*e*), and European *G. holbrooki* had, on average, more sires per brood than Chinese *G. affinis* (Fig. 4.2*c*). Finally, the relationships between environmental PC1 and numbers of sires (*G. holbrooki*: $R^2 = 0.216$; *G. affinis*: $R^2 = 0.068$) as well as between fecundity and numbers of sires (*G. holbrooki*: $R^2 = 0.119$; *G. affinis*: $R^2 = 0.004$) were stronger in *G. holbrooki* than in *G. affinis* (Fig. 4.2*a*, *e*).

European *G. holbrooki* also had higher levels of reproductive skew than Chinese *G. affinis* (Fig. 4.2*d*). Parallel to the pattern uncovered for numbers of sires, reproductive skew increased towards northern sites (i.e., along environmental PC1; Fig. 4.2*b*) and with female fecundity (Fig. 4.2*f*). We did not find significant effects of the interactions 'species × environmental PC1' and 'species × fecundity', suggesting that both species responded similarly to climatic variation and increased reproductive skew similarly with increasing body size/fecundity (Table 4.2*b*).

Table 4.2. General linear models (GLM) on geographical variation in (*a*) numbers of sires and (*b*) reproductive skew in *G. affinis* and *G. holbrooki*, and temporal variation in (*c*) numbers of sires and (*d*) reproductive skew in *G. affinis* from Ankang and Beihai. Statistically significant effects are shown in bold. Non-significant interaction terms that were removed from the final model are shown in brackets.

Dependent variable	Effect	d.f.	*F*	*P*	Partial η^2
(*a*) Geographical variation: Number of sires	**Species**	1	6.147	**0.014**	0.033
	Fecundity	1	19.436	**< 0.001**	0.098
	Environmental PC1	1	24.926	**< 0.001**	0.123
	Environmental PC2	1	1.171	0.281	0.007
	Environmental PC3	1	0.368	0.545	0.002
	Species × fecundity	1	4.403	**0.037**	0.024
	Species × environmental PC1	1	15.688	**< 0.001**	0.081
	[Species × environmental PC2]	1	0.070	0.791	< 0.001
	[Species × environmental PC3]	1	0.039	0.843	< 0.001
	Error	178			
(*b*) Geographical variation: Reproductive skew	**Species**	1	4.616	**0.033**	0.025
	Fecundity	1	15.331	**< 0.001**	0.078
	Environmental PC1	1	4.936	**0.028**	0.027
	Environmental PC2	1	2.994	0.085	0.016
	Environmental PC3	1	0.431	0.512	0.002
	[Species × fecundity]	1	0.011	0.916	< 0.001
	[Species × environmental PC1]	1	0.737	0.392	0.004
	[Species × environmental PC2]	1	1.846	0.176	0.010
	[Species × environmental PC3]	1	0.159	0.691	0.001
	Error	180			
(*c*) Temporal variation: Number of sires	**Population**	1	15.424	**< 0.001**	0.118
	Fecundity	1	10.974	**0.001**	0.087
	Temperature PC	1	0.849	0.359	0.007
	Population × fecundity	1	4.305	**0.040**	0.036
	[Population × temperature PC]	1	0.028	0.868	< 0.001
	Error	115			
(*d*) Temporal variation: Reproductive skew	**Population**	1	8.965	**0.003**	0.072
	Fecundity	1	7.667	**0.007**	0.062
	Temperature PC	1	0.024	0.877	< 0.001
	[Population × fecundity]	1	0.581	0.447	0.005
	[Population × temperature PC]	1	0.092	0.762	0.001
	Error	116			

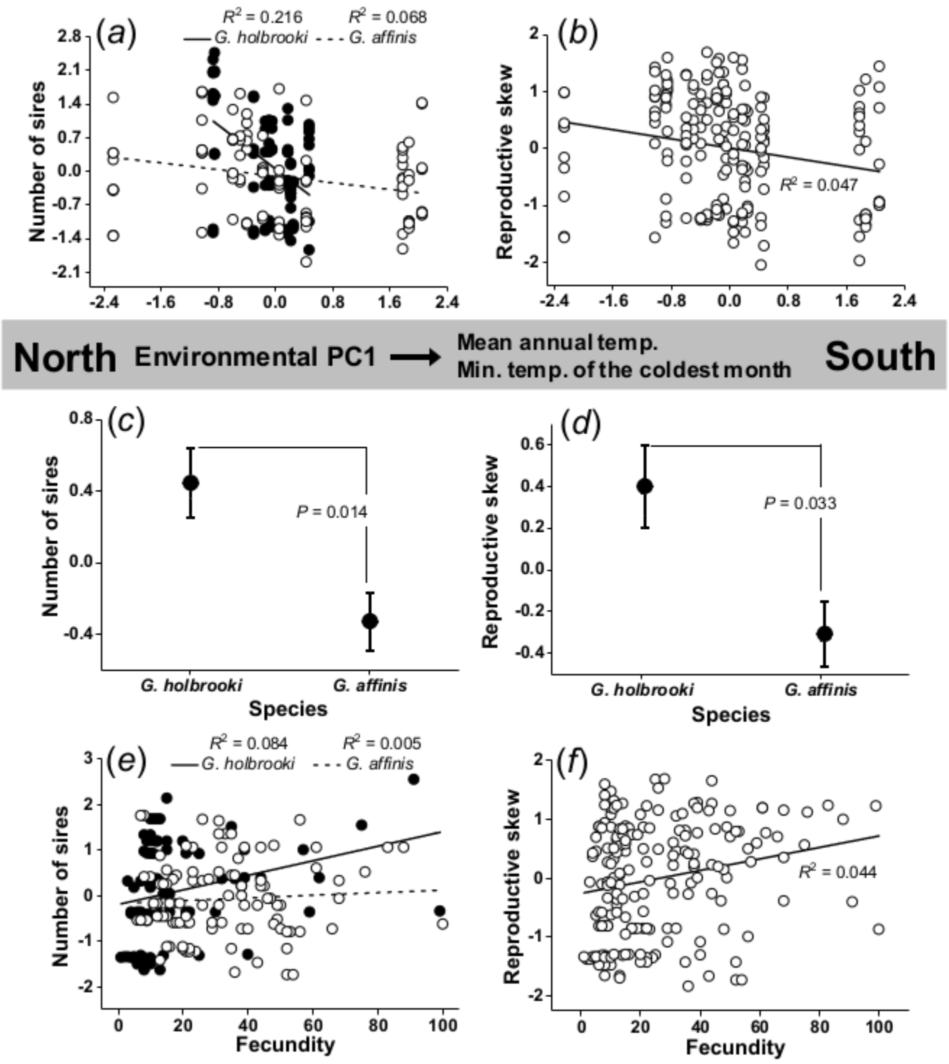

Figure 4.2. Visualization of significant effects from our GLMs on geographic variation in multiple paternity. Depicted are (*a*, *b*) climate-, (*c*, *d*) species- and (*e*, *f*) fecundity-effects on variation in numbers of sires and reproductive skew. In (*a*, *b*, *e*, *f*) are shown residuals, and in (*c*, *d*) estimated marginal means, corrected in both cases for all other model terms. (*a*, *e*): data are presented split by species in order to visualise the significant interaction effects uncovered in the GLMs (see main text and Table 4.2).

Path analysis including effects of climate and population-level life histories

We conducted path analysis to further examine whether the observed pattern of latitudinal variation in MP might reflect indirect climatic effects, mediated by altered life-history traits (assessed on the population level). The single best model identified by our path analysis included 19 out of 32 possible paths (Fig. 4.3), but similar to our GLMs, explanatory power for numbers of sires

($R^2 = 0.15$) and reproductive skew ($R^2 = 0.22$) was low, and model fit was also not particularly strong (GFI = 0.771; $\mathrm{AIC}_c = 544.629$). In the model, all effects of climate on MP were indirect, and only a single direct path from an exogenous variable to a measure of MP (from 'species' to reproductive skew) was retained. The strongest indirect effects of climate on our measures of MP were the positive indirect effects of environmental PC2 onto both number of sires and reproductive skew, indicating that MP increased with increasing distance to the sea, higher annual precipitation and lower annual temperature differences; however, none of these showed consistent, overarching latitudinal patterns in both species. Moreover, these effects were indeed mediated by life-history responses, with relatively weak effects of life-history PC2 (positive) and life-history PC3 (negative) on reproductive skew, and a stronger positive effect of life-history PC4 on numbers of sires. This indicated that reproductive skew increased with increasing fecundity and female SL, as well as increasing male (GSI) and female (RA) investment into reproduction, but also with decreasing embryo lean weight. Similarly, numbers of sires increased with increasing embryo fat content. Even though several other indirect effects of climate on MP were relatively weak, they were nonetheless almost all significant ($P \leq 0.012$).

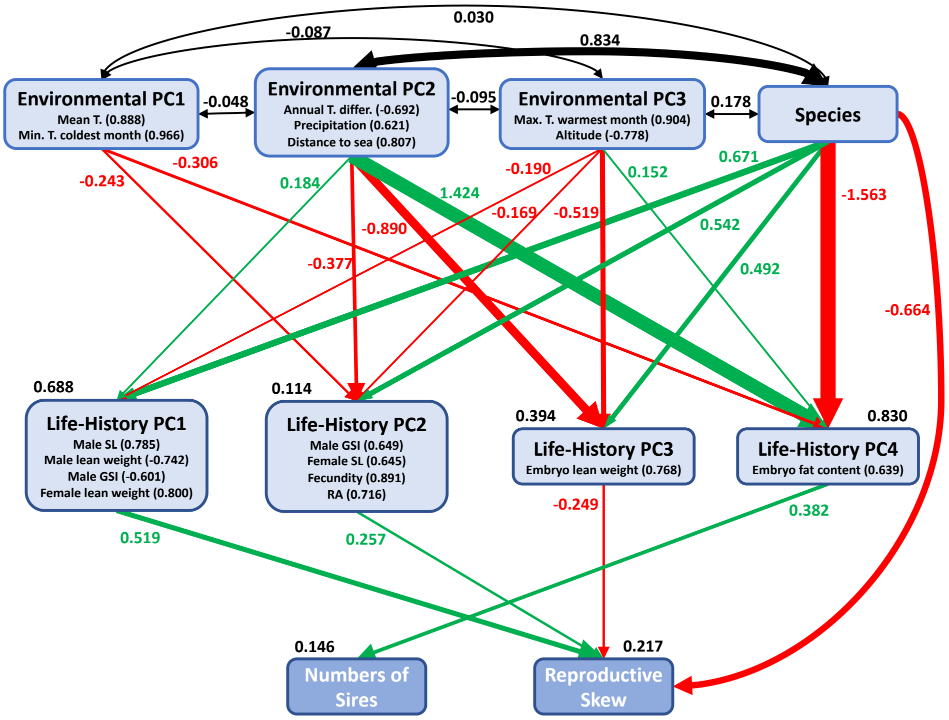

Figure 4.3. Best-fit structural equation model on the influence of environmental variables, species, and life histories on variation in multiple paternity in invasive mosquitofish from Europe and China. Numbers along single-headed arrows represent partial regression coefficients. Green arrows represent positive regression coefficients, while red arrows represent negative ones. Black, double-headed arrows represent covariation between exogenous variables. All arrow dimensions are proportional to the strength of the effect. Next to each endogenous variable is reported its relative R^2. For PCs the variables that have the strongest PC loadings are reported in the boxes.

Temporal variation in MP

GLM results on effects of temperature and individual variation in female fecundity

In the analysis of variation in MP across the reproductive season in *G. affinis* from Ankang and Beihai we found that 'population', fecundity and 'population × fecundity' had significant effects on both estimates of MP (Table 4.2*c*, *d*). Contrary to the analysis of geographical variation and counter to what we predicted *a priori* (Table 4.1), however, we did not find any significant effect of temperature variation on MP (temperature PC; Table

4.2*c*, *d*). Overall, *G. affinis* from Beihai had higher levels of multiple paternity than those from Ankang (Fig. 4.4).

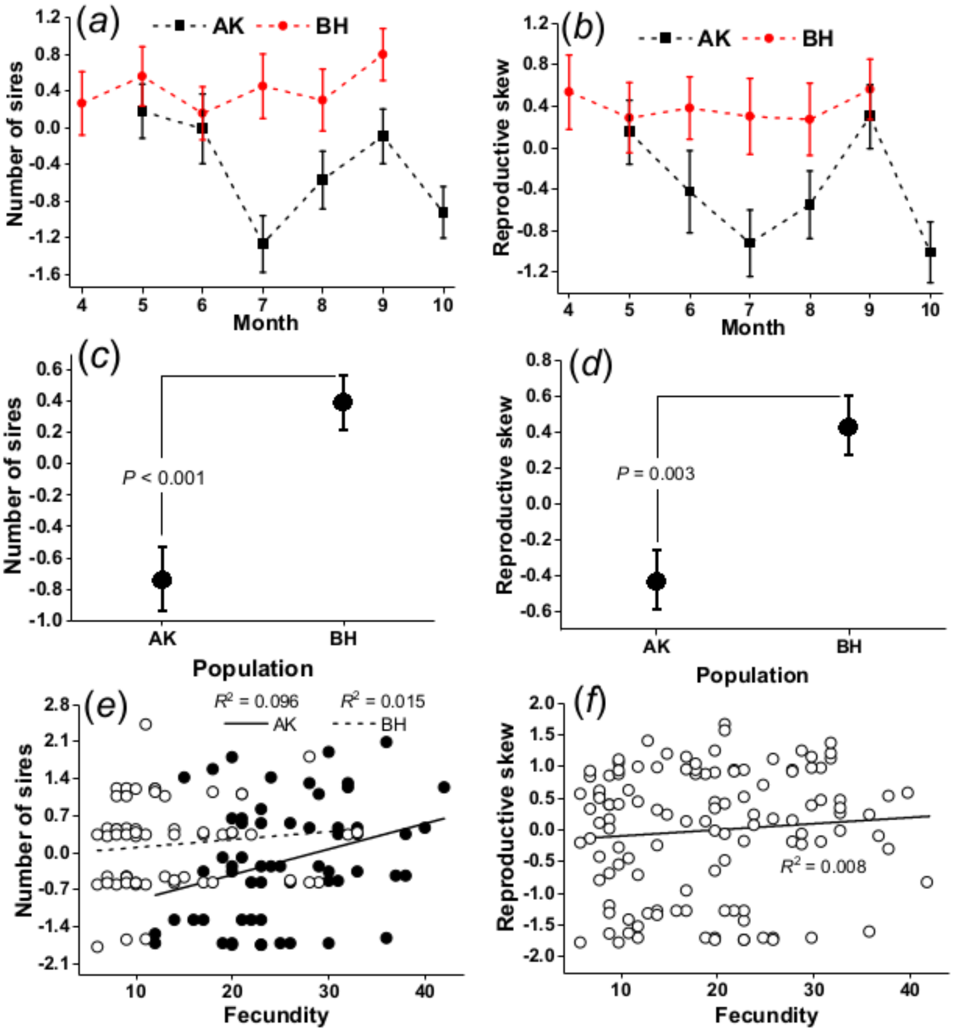

Figure 4.4. Visualization of significant effects from our GLMs on temporal variation in multiple paternity. Depicted are (*a*, *b*) monthly variation, (*c*, *d*) population- and (*e*, *f*) fecundity-effects on MP in *G. affinis* from Ankang (AK) and Beihai (BH). In (*c*, *d*) are shown estimated marginal means, and in (*e*, *f*) residuals, in both cases corrected for all other model terms. (*e*): data are presented split by species in order to visualise the significant interaction effect uncovered in the GLMs (see main text and Table 4.2).

Path analysis including temperature effects, population demography and life histories

Our path analysis on the temporal (i.e., Ankang and Beihai) dataset yielded a model in which one path each remained towards our measures of MP (GFI = 0.839, AIC_c = 228.417; Fig. 4.5). Explanatory power for numbers of sires (R^2 = 0.026) and reproductive skew (R^2 = 0.029) was extremely low, and all the standardized total effects of the temperature PC, ASR and CPUE on number of sires (0.067, 0.101 and -0.027, respectively) and reproductive skew (0.076, -0.035 and 0.086), were weak and were due to indirect effects via life histories, further suggesting that life-history differences are the main drivers of differences in MP (see Table 4.1). Nonetheless, the indirect effects of ASR (P = 0.041) and the temperature PC (P = 0.049) on reproductive skew were significant, while the other indirect effects on our measures of MP bordered significance ($0.057 \leq P \leq 0.071$). Almost all direct effects were significant ($P \leq 0.001$ in all cases), with the exception of the marginally non-significant effects of CPUE on life-history PC2 (P = 0.081), life-history PC2 on numbers of sires (P = 0.078) and life-history PC3 on reproductive skew (P = 0.069). The total effects of temperature on reproductive skew and numbers of sires were weakly positive, indicating that MP tended to increase with increasing temperatures. The total effects of CPUE (population density) on reproductive skew and number of sires were in opposing directions (i.e., negative for numbers of sires and positive for reproductive skew), indicating that numbers of sires decreased with increasing population densities, while reproductive skew increased with increasing population densities. Conversely, the total effects of ASR on reproductive skew and number of sires were also in opposing directions (i.e., positive for numbers of sires and negative for reproductive skew), indicating that numbers of sires increased while reproductive skew decreased with more female-biased sex ratios (Table 4.1). Regarding the effects of life histories, numbers of sires increased with increasing fecundity, but decreased with increasing embryo lean weight (effect of life-history PC2), while reproductive skew increased with increasing embryo fat content (effect of life-history PC3).

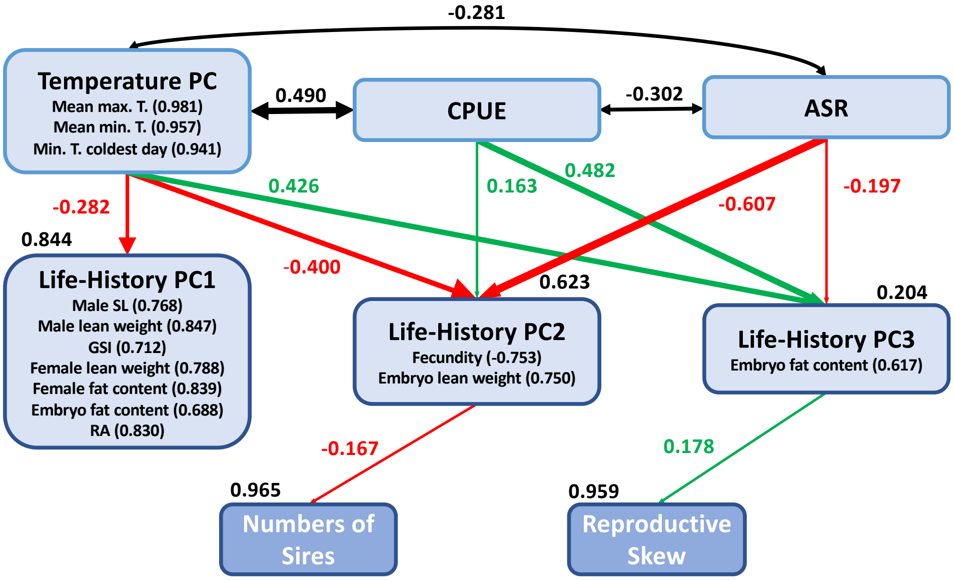

Figure 4.5. Best-fit structural equation model on the influence of climate, demography (population densities, estimated as catch-per-unit-effort, CPUE; adult sex ratios, ASR) and life histories on variation in multiple paternity in invasive *G. affinis* from Ankang and Beihai. Numbers along single-headed arrows represent partial regression coefficients. Green arrows represent positive regression coefficients, while red arrows represent negative ones. Black, double-headed arrows represent covariation between exogenous variables. All arrow dimensions are proportional to the strength of the effect. Next to each endogenous variable is reported its relative R^2. For PCs the variables that have the strongest PC loadings are reported in the boxes.

Discussion

Our analysis on geographical variation of MP showed that both number of sires per clutch and reproductive skew increased in northern populations of both *G. holbrooki* and *G. affinis*. While this result seems to provide support for direct climate-effects, path analysis suggested that climatic effects were mainly indirect, mediated by altered life-history traits, both latitude-dependent and -independent (Ouyang et al. 2018, Riesch et al. 2018). However, both analyses received relatively low statistical support. In the following paragraphs, we will discuss the relative contributions of these effects.

Geographical variation in MP

In the GLMs, MP increased along environmental PC1, which mainly describes variation in mean annual temperature and in minimum temperature of the coldest month across a north-south axis. These responses were slightly different in the two species, as European *G. holbrooki* had a higher number of sires overall and a steeper response to latitudinal variation than Chinese *G. affinis*. Two, non-mutually exclusive factors can be invoked to explain these differences. First, the overall climate gradient between the northern- and southern-most populations we sampled in Europe is much steeper (Δ temperature of warmest month = 6ºC; Δ temperature of coldest month = 7ºC) compared to the climate gradient we sampled in China (Δ temperature of warmest month = 1ºC; Δ temperature of coldest month = 1ºC), even though Chinese populations were sampled across a much wider geographical range

(Δ latitude, Europe = 8.53°, China = 17.3°). Second, we sampled European *G. holbrooki* in July/August, while Chinese *G. affinis* were sampled in April and June. Since reproduction in *Gambusia* is highly seasonal (Pyke 2005), and the temporal analysis of MP revealed that, at least in some cases, MP varies across the reproductive season, temporal variation has to be considered as a possible confounding factor (Cockburn et al. 2008, Kasumovic et al. 2008).

The higher levels of MP at higher latitudes broadly conform to our hypotheses and might suggest that active solicitation of multiple mating by females, but not male coercion, is responsible of this variation (Table 4.1). Indeed, increasing the number of different sires allows each female to increase the genetic variation within broods (Grapputo et al. 2006) and it could serve as a strategy against complete reproductive failure (García-González et al. 2014): by maximising the number of different offspring genotypes (and thus phenotypes), it increases the likelihood for each females that at least some of her offspring would be viable in an unpredictable environment (Mäkinen et al. 2007, Simons 2011, García-González et al. 2014). Likewise, increased reproductive skew in northern populations could indicate higher levels of female-mediated sexual selection in these populations (Emlen and Oring 1977), which could help females to mitigate the negative consequences of sexual harassment and to refine their mate choice (Hamilton 1990, Pitcher et al. 2003), and produce higher-quality offspring (Ojanguren et al. 2005). The idea that female-mediated sexual selection (e.g., via cryptic female choice; Pilastro et al. 2007, Gasparini and Pilastro 2011) increases towards northern population aligns with the results from another study on Chinese *G. affinis*, suggesting that male-mediated forms of sexual selection on certain phenotypic traits increase towards southern populations, where males have a modified armament of their gonopodium tips that may improve sperm transfer during coercive mating (Ouyang et al. 2018).

The results of our path analysis, however, highlighted the presence of latitude-independent effects, and how responses to climate are mostly indirect, mediated by life-history differences (for climatic effects on life histories see also Tökölyi et al. 2014, Lancaster et al 2017, Ouyang et al. 2018, Riesch et al. 2018). The strongest effects of climate on our measures of MP were in fact the positive indirect effects of environmental PC2 onto both number of sires and reproductive skew, indicating that MP increased with increasing distance to the sea, higher annual precipitation and lower annual temperature differences. These patterns were either independent of latitude (distance to sea) or showed opposite patterns in *G. holbrooki* in Europe (higher annual precipitation and lower annual temperature differences in more northern populations) compared to *G. affinis* in China (lower annual precipitation and higher annual temperature differences in more northern populations).

With respect to the effects mediated by life histories, the strong relationship between MP and female body size/fecundity—also confirmed on the individual level in this and in previous studies (Herdman et al. 2004, Deaton 2008, Neff et al. 2008, Zeng et al. 2017)—is important as northern *Gambusia* populations tend to have a higher fecundity (Riesch et al. 2018). Greater female investment into reproduction (RA), and smaller offspring size, was also associated with increased reproductive skew. This could potentially be a signal of cryptic female choice (Evans and Magurran 2001, Pilastro et al. 2007, Gasparini and Pilastro 2011, Magris et al. 2017) becoming more important in populations in which female investment into the current reproductive bout is greater, and thus the potential costs of producing offspring with suboptimal genotypes is likely to be greater as well. Furthermore, increased fecundity was coupled with decreased offspring size (Qualls and Shine 1995). Females could potentially be able to offset any negative effect of reduced offspring size by skewing paternity towards preferred, higher-quality males, thus producing more offspring of higher genetic quality. We lack at present the empirical data to properly dissect these effects. Future studies will need to investigate the effects of (cryptic) female choice in mosquitofish and its geographical variation, especially in relation to the trade-off between offspring size and number.

Lastly, we found that increasing male investment into reproduction (GSI) was associated with greater reproductive skew, which is congruent with the idea that MP is influenced by sperm competition intensity (e.g., Dean et al. 2006).Regarding the increased reproductive skew in northern populations, while it appears tempting to argue in favour of a stronger role for female mate choice (see above; Table 4.1), it seems likely that also in this case life-history effects are primarily responsible for the observed variation. Northern populations are subject to higher overwinter mortality than southern ones (Cheng et al. 2018), leading to lower population densities and female-biased sex ratios, at least at the beginning of the reproductive season (Zulian et al. 1995). While mosquitofish males are, on average, larger at higher latitudes

(Ouyang et al. 2018, Riesch et al. 2018), some males can achieve sexual maturity at particularly small body sizes under these conditions (Zulian et al. 1995; see also Borowski 1978 for *Xiphophorus variatus*), and smaller males are more efficient in sneaky copulations, being more manoeuvrable and more difficult to spot by females (Pilastro et al. 1997).

Temporal variation in MP

When analysing temporal variation in MP, temperature differences failed to provide a strong explanation for the observed monthly variation across the reproductive season in our GLMs. However, in our path analysis (Fig. 4.5), a slightly more nuanced picture emerged.

In contrast to our *a priori* prediction of higher MP levels in colder months (similar to the pattern described for birds by Botero and Rubistein 2012), temperature had a weak, positive effect on MP in our path analysis. However, all standardised effects of climate, adult sex ratio, and CPUE on MP were indirect and mediated by altered life histories, so that numbers of sires increased with increasing fecundity, but decreased with increasing embryo lean weight, while reproductive skew increased with increasing embryo fat content. This analysis further revealed that the effects of increasing population densities and female-biased adult sex ratios were opposite between number of sires—which decreased with increasing population densities and increased with more female-biased sex ratios—and reproductive skew, which increased with increasing population densities and decreased with more female-biased sex ratios. Both effects are further evidence that variation in sperm competition intensity drives parts of the variation in MP (see also Dean et al. 2006). Moreover, our results are congruent with previous studies on *G. holbrooki* reporting that populations with female-biased sex ratios are characterised by smaller males (Zulian et al. 1995) that have higher insemination efficiency in coercive copulations (Pilastro et al. 1997; Table 4.1). We further argue that operational sex ratios (OSR), which we could not assess in the course of this study, may be more meaningful indicators of sperm competition intensities. Alternatively, female *Gambusia* are known to store sperm for several months (Pyke 2005), and—given the relative lack of males at the beginning of the reproductive season—this might have a greater effect in spring than later in the year (i.e., when population densities are lower). Similarly, previous studies in *Gambusia* have shown that males that matured at the end of the reproductive season are bigger than those that matured earlier in the year, and large-bodied males are preferred by females and are able to monopolise access to females (Bisazza and Marin 1991, Zulian et al. 1993, Pyke 2005).

An unexpected result in our monthly sampling data was that throughout 2016, the more southern population (Beihai) had higher levels of MP than the more northern population (Ankang). Moreover, while patterns of MP fluctuated strongly from month to month in Ankang, they remained relatively similar across the reproductive season in Beihai. This is both contrary to the overall geographic trend in both China and Europe in 2017–18, as well as the actual results for both Ankang and Beihai from 2017. We cautiously argue that seasonal and yearly variation in temperature (and subsequently in life-history and demographic traits) might be responsible for such a shift; nevertheless, we cannot exclude that this result could again be influenced by seasonal variation, as the Ankang and Beihai populations were sampled during different months for our geographical analysis. This further supports the idea that differences in MP are—at least to a substantial extent—explained by indirect, not direct effects of climate and temperature.

Altogether then, no single selective agent received unanimous support as the main driver of MP across our different analyses. Thus, it appears as if patterns of MP in natural populations of invasive mosquitofish are the result of multiple selective forces that interact in a complex manner, and while there seem to be some overarching patterns (i.e., a general increase in MP with latitude), patterns are also to some degree specific to each species, and clearly differ from population to population. Moreover, the relatively low explanatory power of our models suggests that additional variables we did not quantify also contribute to patterns of MP. Likely candidates include additional sources of variation in sexual selection, such as predation (Kelly et al. 1999, Plath et al. 2019) or water pollution (xenestrogens: Díez-del-Molino et al. 2018), as well as several other environmental factors (see Table 4.1 for a breakdown of factors known to influence MP in poeciliid fishes). However, it is also possible that slightly different climatic variables (for the geographic study, for example, averages of the actual year of collection rather than multi-year averages) would have increased predictive power in our analyses. Finally, our argumentation hinges on the assumption that life-history traits influence patterns of MP, when some work has shown that causality could also be reversed. For example, higher rates of MP could result in higher fecundities (Noble et al. 2013). Clearly, more work is needed to fully disentangle the main drivers of MP in natural populations.

Consequences for the invasiveness of Gambusia *spp.*

Independent of the mechanisms behind the observed patterns of geographic and temporal variation in MP, our results show that invasive *Gambusia* adjust levels of MP in response to both environmental/climatic and life-history/demographic factors. Along latitudinal gradients, the amount of MP increased with latitude. In both the native and the invasive distribution ranges, northward expansions of *Gambusia* are mainly limited by low temperatures (Benejam et al. 2009, Riesch et al. 2018), while southern populations have often reached some geographical barrier that prevents further range expansion towards the South (the Mediterranean Ocean and Gulf of Mexico/Atlantic Ocean for *G. holbrooki* in their invasive and native range, respectively, and the South China Sea for *G. affinis* in China). Thus, the greatest potential for future range expansion is along the northern distribution edges in both invasive ranges, and it is exactly here that both species of *Gambusia* show the highest levels of MP. Moreover, female *Gambusia* can store sperm for several months (Pyke 2005), and a single mated poeciliid female is potentially sufficient to found a new, viable population (Deacon et al. 2014). Therefore, even though conflicting hypotheses on the mechanisms behind variation in MP (Table 4.1) could not be resolved unambiguously, our present study suggests that, in light of the predicted increases in temperature due to Global Climate Change (Rojeli and Knutti 2016, Carboni et al. 2017), invasiveness and further northward range expansion will likely be boosted by the high levels of MP in northern populations of these species.

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Data accessibility

The data that support the findings of this study are openly available in DRYAD at https://doi:10.5061/dryad.kprr4xh0r.

Authors’ contributions

MP conceived the study, and JG, FS, RR and MP designed the study. All authors collected the data. JG, FS and RR performed data analyses. FS wrote the first draft of the manuscript. All authors contributed to manuscript writing and approved the final version.

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Chapter 5: Phenotypic responses to natural and anthropogenic oil pollution in Trinidadian guppies (*Poecilia reticulata*).

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Abstract

Humans are among the strongest drivers of environmental change, as they impact ecosystems in several ways. One of the most important human impacts is pollution, including that arising from exploitation of crude-oil reserves in oil fields. While pollution often leads to population collapses and reduced biodiversity, several species have been able to colonise polluted environments and evolve genetic adaptations to oil pollution. Here, we used guppies (*Poecilia reticulata*) from southern Trinidad, where oil pollution is widespread, to analyse morphological and life history responses to crude oil. We used N = 355 individuals from 11 independent populations, 6 living in polluted environments, and 5 living in non-polluted ones. Overall, guppies from oil-polluted habitats were characterised by increased body size; rounder, deeper bodies with increased head size; and increased offspring size when compared with their counterparts from non-polluted sites. Our results, together with the high amount of population-specific phenotypic diversity, paint a complicated picture that suggests that guppies might not only be subject to the direct negative effects of oil pollution, but could also be (indirectly) positively affected by reduced predation and parasite load. This further highlights the exceptional ability of this species to colonise and thrive in degraded environments.

Key-words

Poecilia reticulata, oil pollution, life history, body shape

Introduction

Humans are a major source of environmental change (Palumbi 2001, Hendry et al. 2017). We impact ecosystems through climate change, habitat destruction, introduction of invasive species and pollution (Vitousek et al. 1997, Palumbi 2001, Stockwell et al. 2003, Pelletier and Coltman 2018). While these processes are typically considered as negative, as they lead to loss of biodiversity (Forester and Machlis 1996), recently they have also been recognised as an important factor promoting rapid phenotypic and genetic differentiation (Palumbi 2001, Hendry et al. 2008, Palkovacs et al. 2012, Hendry et al. 2017), even leading to speciation (Bull and Maron 2016). Among the anthropogenic sources of environmental variation, pollution is one of the most important, and is one of the main drivers of contemporary evolution (Hamilton et al. 2017, Hendry et al. 2017).

Fish are especially susceptible to the negative effects of pollution (Ricciardi and Rasmussen 1999, Harmon and Wiley 2009), experiencing both lethal and sublethal effects (such as immunodepression and increased parasitism, Poulin 1992; Austin 2010; or reduced reproduction, Kime 1995). Nevertheless, numerous fish species have been able to colonise both naturally-toxic environments (Schelkle et al. 2012, Riesch et al. 2015, Tobler et al. 2018) and environments that are toxic as a result of human activities (Hamilton et al. 2017). In several cases, fish have evolved adaptations to toxic chemicals (Laporte et al. 2016, Reid et al. 2016, Hamilton et al. 2017). For example, multiple populations of the killifish *Fundulus heteroclitus* and *F. grandis* across the U.S.A. have evolved repeated genomic adaptation in response to hydrocarbons and dioxin-like pollutants (Whitehead et al. 2011, 2012). On the other hand, many species seem unable to adapt to high levels of pollution, resulting in reduced population sizes (Rolshausen et al. 2015), and in reduction of overall biodiversity in affected habitats (Johnston and Roberts 2009). There are still important gaps in our understanding of the traits essential for fish to tolerate high levels of pollution, and of the associated costs, for instance in terms of fitness and/or life-history trade-offs (Hamilton et al. 2017).

Among pollutants, crude oil is recognised as having widespread, deleterious effects on wildlife (Wake 2005). The effects of oil pollution on ecosystems have most often been studied in the case of large-scale disasters, such as the Exxon Mobil (Peterson et al. 2003) or the Deep Water Horizon incidents (Barron 2012, White et al. 2012). However crude oil can also enter the environment at a smaller scale through spillages that occur during the exploitation of crude-oil reserves in oil fields (Wake 2005, Kelly et al. 2010, Rolshausen et al. 2015), creating highly toxic environments that impact only certain populations of a species (Rolshausen et al. 2015).

Once released into the environment, crude oil has several lethal and sublethal negative effects upon exposed biota (Wake 2005, Hamilton et al. 2017), which have been documented in multiple species of fish (Irwin 1965, Saha and Konar 1984a, b, Rowe et al. 1983a). For example, in a study on the effects of high concentration of crude oil on *Oreochromis mossambicus*, most individuals died within 24h of exposure (Saha and Konar 1984a). Even at much lower crude oil concentrations, fish experienced respiratory distress, hampered growth (Saha and Konar 1984b), and reduced fecundity (Rowe et al. 1983a). Moreover, one of the main components of crude oil, polycyclic aromatic hydrocarbons, have well-established carcinogenic and mutagenic effects on organisms (Pickering et al. 1989, Samanta et al. 2002).

Trinidadian guppies, *Poecilia reticulata*, are among the few species that can tolerate high levels of water pollution (Irwin 1965, Araújo et al. 2009, Riesch et al. 2016, Gomes-Silva et al. 2019). They have also been the subject of decades of ecological and evolutionary studies (Magurran 2005), and their biology is well-understood, making them an ideal model for the study of phenotypic responses to pollution in aquatic ecosystems. In southern Trinidad oil pollution is widespread as a consequence of human exploitation of crude-oil reserves, and guppies have been able to colonise several highly polluted environments (Rolshausen et al. 2015). Guppies can also be found in the Pitch Lake, the largest natural asphalt lake in the world (Schelkle et al. 2012), where crude oil and bitumen seep to the surface creating a highly toxic environment (Ponnamperuna and Pering 1967, Santi et al. 2019).

Here, we analysed morphological and life-history responses to oil pollution in guppies from six polluted and five non-polluted sites across mostly southern Trinidad. While a previous study failed to uncover evidence of local adaptation in response to oil pollution in two populations from anthropogenically-polluted sites in southern Trinidad (Rolshausen et al. 2015), guppies have been known to undergo rapid phenotypic and genetic changes in response to varying environmental conditions, such as differences in predation (Reznick and Endler 1982, Reznick et al. 1990, Reznick et al. 1997) or fishing pressures (van Wijk et al. 2013). Even without the presence of local adaptation to oil pollution, guppies exhibited parallel divergence in body shape in polluted environments (Rolshausen et al. 2015), with fish from oil-polluted environments being characterised by shallower bodies but bigger and deeper heads than those from non-polluted environments (Rolshausen et al. 2015). Furthermore, in a preliminary study on life-history responses of guppies from the Pitch Lake compared to a nearby, non-polluted site, we found that Pitch Lake guppies were characterised by *r*-selected life histories, with increased reproductive investment and fecundity, and reduced body size and offspring size (Santi et al. 2019). These results suggest that phenotypic responses, even if only mediated by phenotypic plasticity, might be important in the colonization of polluted environments (Ghalambor et al. 2007).

Based on these previous results, which suggest a scenario of increased mortality caused by oil pollution, we made the following *a priori* predictions: (1) guppies across polluted environments should be characterised by increased head size and shallower bodies than their counterparts from non-polluted sites (Rolshausen et al. 2015; see also Tobler and Hastings 2011, Riesch et al. 2016). Moreover, (2) guppies living in oil-polluted environments should follow similar life-history strategies to those from the Pitch Lake (namely: reduced body size, increased reproductive investment and increased fecundity coupled with smaller offspring size; Santi et al. 2019).

Materials and Methods

Sampling sites

We sampled guppies from 11 populations across southern Trinidad in June 2018 (Fig. 5.1*a*, *c*). In each population, we sampled 30 mature-looking males and 30 pregnant-looking females. We checked sexual maturation and pregnancy status during life-history dissections (see below) on the basis of gonopodium morphology in males and of the presence of developing embryos in females (Kelly et al. 2000), leading to a final sample size of 355 individuals (201 sexually mature males, 154 pregnant females; see Tables D.1, D.2).

Six populations originated from oil-polluted habitats (identified by the presence of an oil slick on the water surface) and five from non-polluted ones (see Fig. 5.1*b*, *d* for a comparison between oil-polluted and non-polluted habitats). The polluted habitats were the Pitch Lake, where pitch and oil naturally seep to the surface leading to a highly-toxic environment (Richardson 1912, Ponnamperuna and Pering 1967), and five streams subject to crude oil spillage of anthropogenic origin, chosen in order to have a good representation of the several streams flowing through the oil fields of southern Trinidad (Rolshausen et al. 2015). As non-polluted sampling sites we chose non-polluted streams closest to the polluted habitats in which we were able to catch adequate numbers of guppies. A population from the north range of the island was included as a further comparison. All sampling sites were small natural and artificial (i.e., ditches) aquatic habitats with stagnant-to-slow-moving water. All fish were sampled with hand-held seines and dip nets, euthanised with clove oil immediately after collection (Fernandes et al. 2017), and then fixed and preserved in 90% ethanol for subsequent analyses. During sampling, we recorded latitude and longitude of each habitat using a Garmin GPSMAP 64s (Garmin Ltd., Olathe, Kansas, USA) and documented all the species that were observed. We also measured water temperature [ºC], dissolved oxygen [mg L^{-1}], salinity [ppt] and pH using a Hach Rugged Field Kit (Hach, Loveland, Colorado, USA), (see Table D.3). Preliminary screening did not reveal any significant influence of these abiotic environmental factor on guppy phenotypes, so they were excluded from the final analyses.

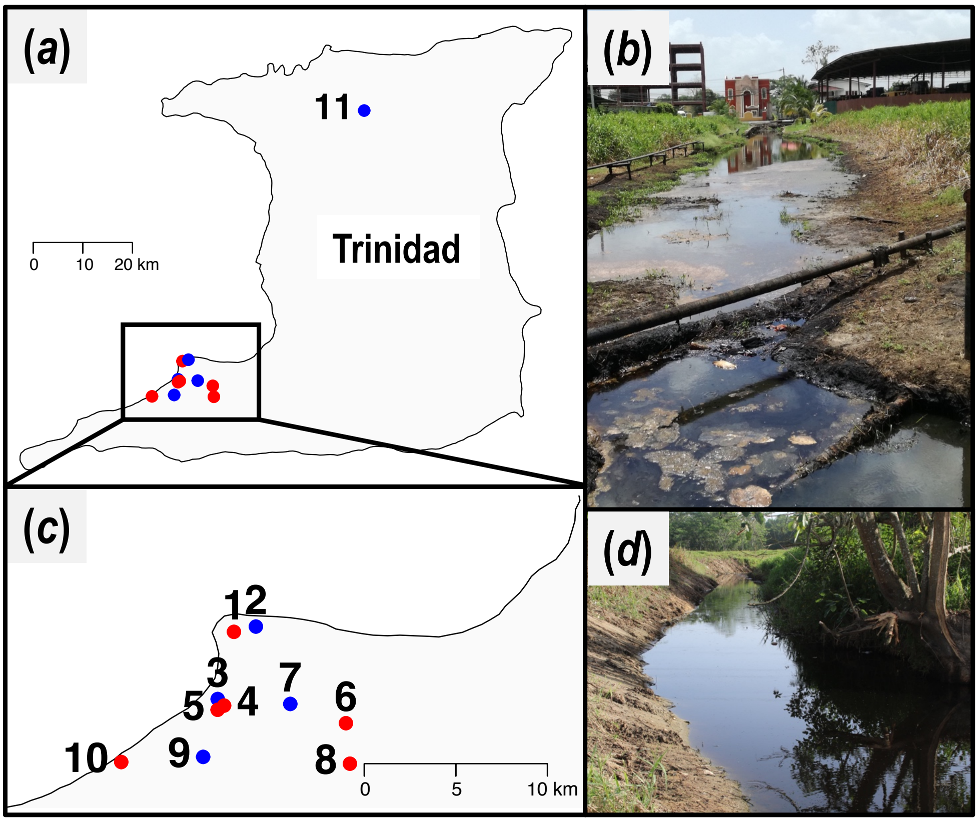

Figure 5.1. Overview of guppy sampling sites in Trinidad. (*a*), (*c*) Map of Trinidad with the location of the 11 sampling sites. Red dots: polluted environments; blue dots: non-polluted environments. (*b*) Example of oil-polluted environment (population 11); (*d*) example of non-polluted environment (population 7). (*a*), (*c*) were created with the R package Maps v. 3.30 (Becker et al. 2018); (*b*), (*d*) photos by F. Santi.

Body-shape analyses

We used geometric morphometrics to analyse fish body shape (Rohlf and Marcus 1993, Zelditch et al. 2012). For each fish, we took standardised photographs of the left side of the body using a Canon EOS 400D DSLR camera with a 50 mm macro lens (Canon Inc., Tokyo, Japan) mounted on a copy stand. All photos were collated into a .TPS file using tpsUtil32 V1.70 (Rohlf 2016a), and 15 landmarks were added to each photo using tpsDig232 V2.26 (Rohlf 2016b). Landmarks were as follows: (1) tip of the upper lip, (2) anterior and (3) posterior insertion of the dorsal fin, (4) top, and (5) bottom of the caudal peduncle, (6) posterior and (7) anterior insertion of the anal fin, (8) pelvic fin, (9) where the ventral end of the operculum meets the body, (10) dorsal end of the operculum, (11) anterior margin of the eye orbit, (12) centre of the eye, (13) posterior margin of the eye orbit, (14) dorsal and (15) ventral insertion of the pectoral fin (following Santi et al. 2020). As some ($N = 29$) individuals became bent during fixation and preservation, we used the "unbend specimen" function in tpsUtil32, which employs quadratic regression to correct the bending effects. To that end, three temporary landmarks were added along the lateral line of the fish and in the middle of the caudal peduncle, and were subsequently removed from the final analysis (Ouyang et al. 2018).

We then performed a relative warp analysis (Zelditch et al. 2012) using tpsRelW32 V2.26 (Rohlf 2016c). We obtained 5 relative warps (RWs) that described 90.95% of the total body-shape variation (Table D.4). Visual representation using thin-plate splines showed that RW1 mainly described differences between males and females, caused by the different positioning of the anal fin, which is modified into the gonopodium, and is shifted towards the anterior in males and towards the posterior in females. RW2-5 on the other hand described differences in the depth and roundness of the body, in the depth of the caudal peduncle, and in head size (Fig. D.1). We used these RWs as shape variables for all subsequent analyses, together with centroid size (i.e., the sum of the quadratic distances of each landmark from their centroid) which was used as a covariate in those analyses to control for body-size effects.

Life-history analyses

We quantified life-history traits by dissecting the fish following well-established life-history protocols (Reznick and Endler 1982, Riesch et al. 2016). We measured the following male and female life-history traits: standard length (SL [mm]), dry weight [mg], lean weight [mg] (dry weight after fat extraction) and fat content [% of dry weight], as well as male gonadosomatic index (GSI [%]; testis dry weight divided by the sum of somatic and testis dry weight), female fecundity (number of developing embryos), and female reproductive allocation (RA [%]; total offspring dry weight divided by the sum of maternal somatic and total offspring dry weight), offspring dry weight [mg], offspring lean weight [mg], and offspring fat content [%] (descriptive statistics of life-history traits are reported in Tables D.1, D.2). We further assessed the developmental stage of each embryo following Riesch et al. (2011), with embryonic stages ranging from 2 (fertilized oocyte with blastodisc present) to 50 (embryo ready to be born), and used embryonic stage of development as a covariate in order to control for developmental effects in subsequent analyses.

In order to meet statistical assumptions of normality of residuals, we log10-transformed (SL, adult lean weight and offspring lean weight), square root-transformed (fecundity), or arcsine(square root)-transformed (GSI, RA, adult and embryo fat content) all life-history variables. We subsequently *z*-transformed all variables to obtain unit-free variables with equal variance for all subsequent analyses.

Statistical analyses

We firstly investigated body-size differences between habitat types (i.e., polluted vs. non-polluted environments) and populations by performing a nested univariate GLM (general linear model) with ‘SL’ as the dependent variable and ‘sex’, ‘habitat type’, and ‘population-nested-within-habitat type’ (henceforth ‘population(habitat type)’) as factors.

We then analysed body-shape differences by running a multivariate GLM with RW1-5 as dependent variables, ‘sex’, ‘habitat type’ and ‘population(habitat type)’ as factors, and ‘centroid size’ as a covariate.

Lastly, we investigated life-history variation by running two separate multivariate GLMs, one on adult and one on offspring-related life-history traits. In the GLM on adult life histories, we used lean weight, fat content, and investment into reproduction (GSI for males and RA for females) as dependent variables, ‘sex’, ‘habitat type’, and ‘population(habitat type)’ as factors, and ‘SL’ as a covariate; whereas in the GLM on offspring-related life histories we used fecundity, offspring lean weight, and offspring fat content as factors, ‘habitat type’ and ‘population(habitat type)’ as factors, and ‘female SL’ and ‘embryonic stage of development’ as covariates. In all analyses, we initially entered all interaction terms in the model, then subsequently removed those with $P > 0.1$ from the final model. All analyses were performed with IBM® SPSS® Statistics for Windows V.25 (2019; IBM Corp. Armonk, NY).

Results

Body-size variation

In the analysis of male and female body-size variation, we found significant effects of the factors ‘sex’ ($F_{1, 333} = 1428.40$, $P < 0.001$, $\eta_p^2 = 0.811$), ‘habitat type’ ($F_{1, 333} = 54.52$, $P < 0.001$, $\eta_p^2 = 0.141$, Table 5.1), and ‘population(habitat type)’ ($F_{9, 333} = 25.64$, $P < 0.001$, $\eta_p^2 = 0.409$), as well as of the interaction ‘sex × population(habitat type)’ ($F_{9, 334} = 3.88$, $P < 0.001$, $\eta_p^2 = 0.143$; Table 5.2). Females were bigger than males (‘sex’-effect), even though overall body size, as well as the extent of sexual dimorphism, varied between populations within each habitat type (‘population(habitat type)’- and ‘sex × population(habitat type)’-effect, respectively). Guppies were characterised by greater SL in polluted environments than in non-polluted ones (Fig. 5.2). While this response appeared to be on average stronger in females ($\Delta SL = 1.6$) than in males ($\Delta SL = 0.7$), the interaction ‘sex × habitat type’ was not significant ($F_{1, 333} = 3.52$, $P = 0.062$).

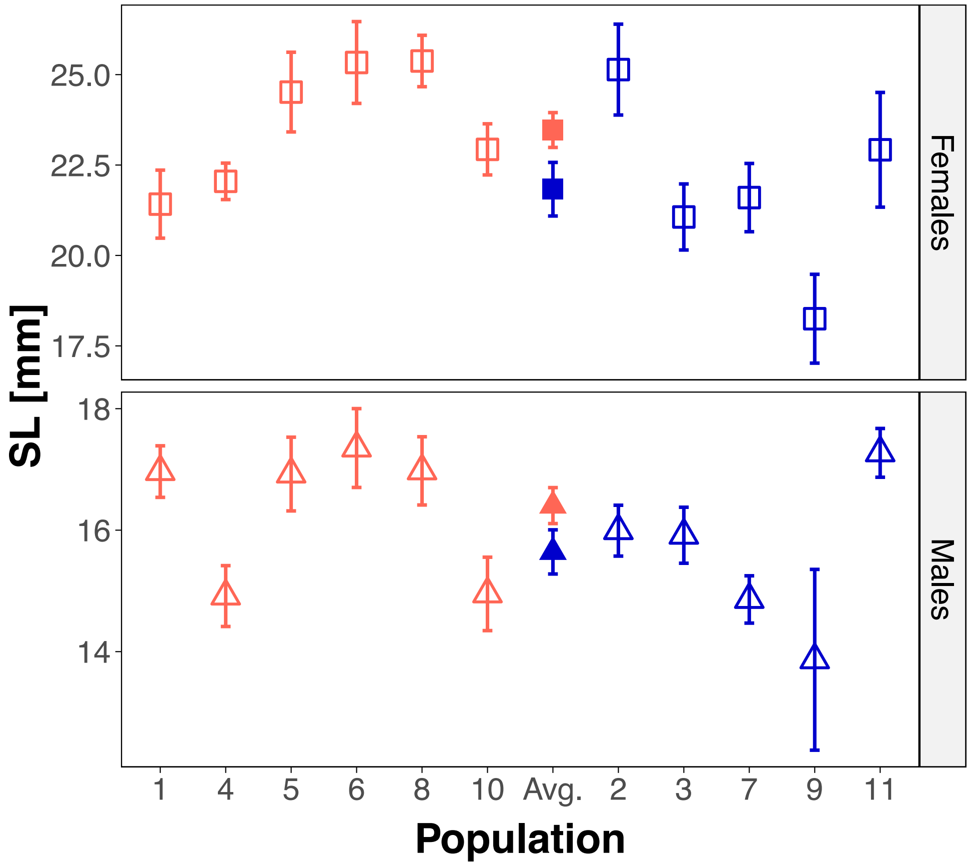

Figure 5.2. Body-size variation (mean ± SE) between populations of guppies living in polluted and non-polluted habitats. Red: polluted environments; blue: non-polluted environments; squares: females; triangles: males. Filled symbols show the average (Avg.) across all polluted and all non-polluted environments.

Table 5.1. Univariate general linear model (GLM) on guppy SL variation between sexes, habitat types (polluted vs. non-polluted), and populations. Significant effects are highlighted in bold.

Factor	*F*	Degrees of freedom	*P*	Partial η^2
Sex	1428.404	1, 333	**< 0.001**	0.811
Habitat type	54.519	1, 333	**< 0.001**	0.141
Population(habitat type)	25.639	9, 333	**< 0.001**	0.409
Sex × habitat type	3.518	1, 333	0.062	0.010
Sex × population(habitat type)	3.881	9, 333	**< 0.001**	0.143

Body-shape variation

The GLM revealed significant effects of 'centroid size', 'sex', 'habitat type', 'population(habitat type)', as well as of the interactions 'sex × habitat type' and 'sex × population(habitat type)' (Table 5.2*a*). Based on our estimate of effect size (partial η^2), 'sex' had the strongest effect ($\eta_p{}^2 = 0.844$), followed by already relatively weak effects of 'centroid size', 'habitat type', and 'population(habitat type)' ($\eta_p{}^2 = 0.177$, 0.167, and 0.152, respectively), while the interactions had even weaker effects ($\eta_p{}^2 < 0.1$). *Post-hoc* univariate GLMs (α-level corrected for multiple comparisons with $\alpha' = 0.010$; Table 5.3) revealed that 'centroid size' and 'sex' significantly affected RW1, RW3 and RW5. 'Habitat type' had significant effects on RW1, RW2, and RW4, while RW1-3 and RW5 were affected by 'population(habitat type)'. Lastly, the interaction 'sex × habitat type' had a significant effect on RW2 and RW3, while 'sex × population(habitat type)' affected RW1-3.

Both overall body shape, as well as the extent of sexual dimorphism varied between populations within the same habitat type ('population(habitat type)' and 'sex × population(habitat type)'-effects). Nonetheless guppies also exhibited consistent responses to the presence of oil pollution, with fish from polluted habitats being characterised by rounder, deeper bodies with relatively larger heads than fish from non-polluted sites ('habitat type'-effect; Fig. 5.3), and these responses were much more pronounced in females than in males ('sex × habitat type'-effect; Fig. 5.3).

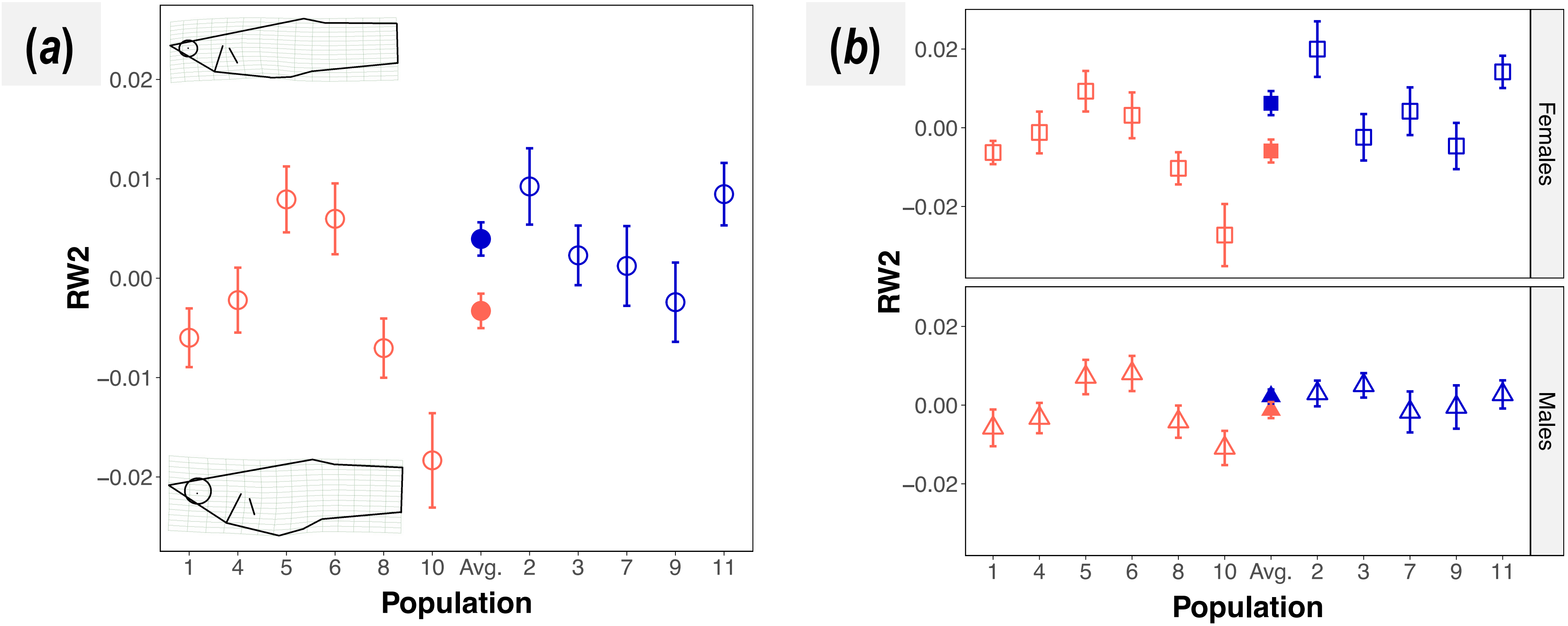

Figure 5.3. Body-shape (RW2) variation (mean ± SE) in guppy populations living in polluted and non-polluted habitats. Body-shape differences are visualised using thin-plate splines. (*a*): overall effect; (*b*) sex-specific effects. Red: polluted environments; blue: non-polluted environments. Squares: females; triangles: males. Filled symbols show the average (Avg.) across all polluted and all non-polluted environments.

Life-history variation

In the analysis of adult life-history traits, we found significant effects of the covariate 'SL', the factors 'sex' and 'population(habitat type)', as well as the interactions 'sex × population(habitat type)' and 'SL × population(habitat type)'. 'Habitat type' did not have a significant effect on adult life histories, but was close to significance ($P = 0.057$; Table 5.2*b*). *Post-hoc* GLMs ($\alpha' = 0.017$; Table 5.4) revealed a significant effect on lean weight by all factors ($P < 0.001$ in all cases) except 'habitat type' ($P = 0.018$). GSI/RA were affected by 'sex' and 'population(habitat type)' ($P < 0.001$ in both cases), while fat content was not affected by any factor or covariate. Lean weight increased with 'SL', and females were on average heavier than males. Moreover, the 'population(habitat type)'-effect, as well as the effects of both interactions, highlighted high levels of differentiation between populations, independently from habitat type. For instance, in polluted habitats, female lean weight ranged from 0.085 ± 0.004 g (mean ± SE) in population 8 to 0.037 ± 0.002 g in population 1, while, in non-polluted habitats, it varied between 0.076 ± 0.005 g in population 2 and 0.032 ± 0.004 in population 9 (Table D.2).

Regarding our analysis of offspring-related life-history traits, 'SL', 'habitat type', and 'population(habitat type)' had significant effects , while 'embryonic stage of development' had again a marginally non-significant effect ($P = 0.068$; Table 5.2*c*). *Post-hoc* GLMs ($\alpha' = 0.017$; Table 5.5) showed that 'SL' significantly affected fecundity, 'habitat type' had a further significant effect on embryo lean weight, and 'population(habitat type)' had a significant effect on all life-history traits analysed. Bigger females produced more offspring than smaller ones ('SL'-effect), and females in oil-polluted environments produced heavier offspring than in non-polluted ones ('habitat type'-effect; Fig. 5.4). Nonetheless, significant variation in offspring life-history traits was again present between populations within habitat type. For instance, fecundity ranged from 15.64 ± 1.44 (population 4) to 9.00 ± 0.82 (population 1) in polluted habitats, and from 27.14 ± 2.59 (population 2) to 5.86 ± 0.67 (population 12) in non-polluted ones (Table D.2).

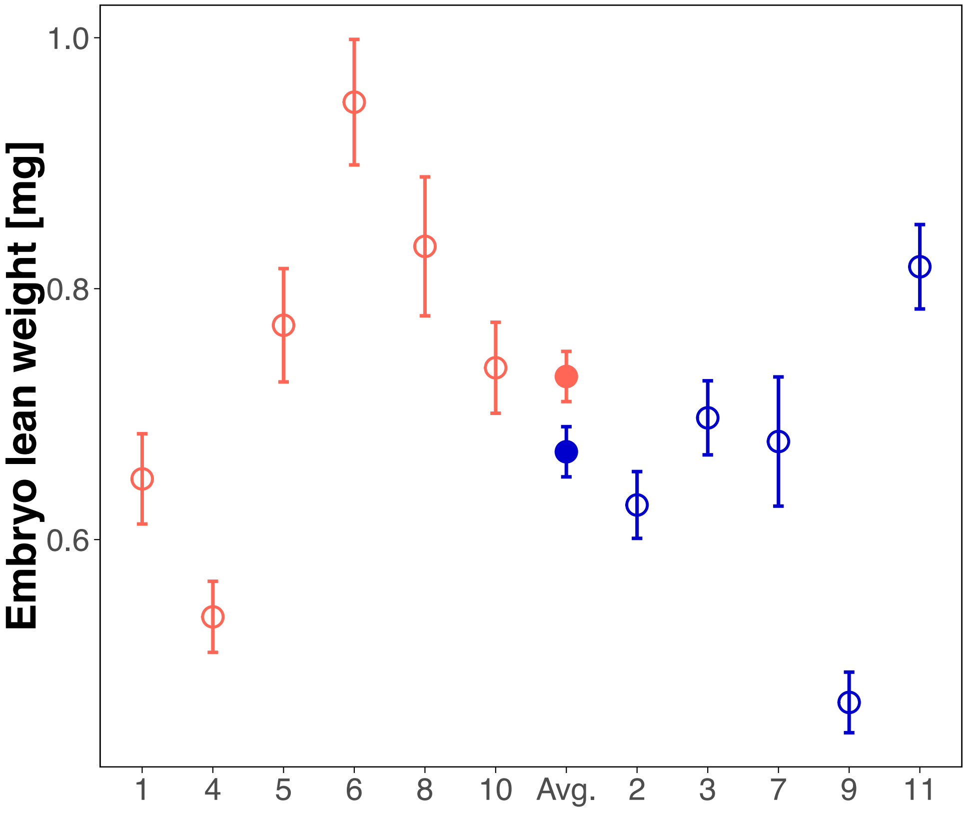

Figure 5.4. Embryo lean weight variation (mean ± SE) in guppy populations living in polluted and non-polluted habitats. Red: polluted environments; blue: non-polluted environments. Filled symbols show the average (Avg.) across all polluted and all non-polluted environments.

Table 5.2. Multivariate general linear models (GLMs) on guppy phenotypic variation. (*a*) male and female body shape, (*b*) adult life-history traits, and (*c*) offspring life-history traits. Significant effects are highlighted in bold.

	Factor	*F*	Degrees of freedom	*P*	Partial η^2
(*a*) male and female body shape	Centroid size	14.708	5, 328	**< 0.001**	0.183
	Sex	407.960	5, 328	**< 0.001**	0.861
	Habitat type	13.1340	5, 328	**< 0.001**	0.169
	Population(habitat type)	6.868	45, 1470	**< 0.001**	0.157
	Sex × habitat type	4.130	5, 328	**0.001**	0.059
	Sex × population (habitat type)	2.736	45, 1470	**< 0.001**	0.068
(*b*) adult life-history traits	SL	299.772	3, 320	**< 0.001**	0.738
	Sex	72.913	3, 320	**< 0.001**	0.406
	Habitat type	2.527	3, 320	0.057	0.023
	Population(habitat type)	8.765	27, 935	**< 0.001**	0.197
	Sex × population (habitat type)	2.751	3, 320	**< 0.001**	0.079
	SL × population (habitat type)	2.207	30, 940	**< 0.001**	0.064
(*c*) offspring life-history traits	SL	20.386	3, 126	**< 0.001**	0.327
	Embryo stage	2.434	3, 126	0.068	0.055
	Habitat type	3.752	3, 126	**0.013**	0.082
	Population(habitat type)	6.387	27, 369	**< 0.001**	0.312
	SL × population (habitat type)	1.472	30, 371	0.055	0.104

Table 5.3. *Post-hoc* univariate GLMs on guppy body shape variation. α-levels have been corrected for multiple comparisons with α' = 0.010. Significant effects are highlighted in bold.

Factor	Dependent variable	*F*	Degrees of freedom	*P*	Partial η^2
Centroid size	**RW1**	37.703	1, 332	**< 0.001**	0.102
	RW2	0.331	1, 332	0.773	0.001
	RW3	24.559	1, 332	**< 0.001**	0.069
	RW4	0.051	1, 332	0.779	< 0.001
	RW5	13.361	1, 332	**< 0.001**	0.039
Sex	**RW1**	1657.197	1, 332	**< 0.001**	0.833
	RW2	0.327	1, 332	0.568	0.001
	RW3	19.125	1, 332	**< 0.001**	0.054
	RW4	0.000	1, 332	0.993	< 0.001
	RW5	16.660	1, 332	**< 0.001**	0.048
Habitat type	**RW1**	13.045	1, 332	**< 0.001**	0.038
	RW2	25.223	1, 332	**< 0.001**	0.071
	RW3	5.814	1, 332	0.016	0.017
	RW4	11.325	1, 332	**0.001**	0.033
	RW5	0.262	1, 332	0.609	0.001
Population (habitat type)	**RW1**	6.008	9, 332	**< 0.001**	0.140
	RW2	11.216	9, 332	**< 0.001**	0.223
	RW3	11.204	9, 332	**< 0.001**	0.223
	RW4	2.074	9, 332	0.031	0.053
	RW5	2.606	9, 332	**0.006**	0.066
Sex × habitat type	RW1	1.902	1, 332	0.169	0.006
	RW2	8.310	1, 332	**0.004**	0.024
	RW3	9.312	1, 332	**0.002**	0.027
	RW4	0.007	1, 332	0.935	< 0.001
	RW5	1.268	1, 332	0.261	0.004
Sex × population (habitat type)	**RW1**	3.333	9, 332	**0.001**	0.083
	RW2	3.714	9, 332	**< 0.001**	0.091
	RW3	4.362	9, 332	**< 0.001**	0.106
	RW4	1.277	9, 332	0.248	0.033
	RW5	1.518	9, 332	0.140	0.040

Table 5.4. *Post-hoc* univariate GLMs on adult life-history trait variation. α-levels have been corrected for multiple comparisons with $\alpha' = 0.017$. Significant effects are highlighted in bold.

Factor	Dependent variable	*F*	Degrees of freedom	*P*	Partial η^2
SL	**Lean weight**	892.080	1, 322	**< 0.001**	0.735
	Fat content	3.508	1, 322	0.062	0.011
	GSI/RA	1.196	1, 322	0.275	0.004
Sex	**Lean weight**	30.370	1, 322	**< 0.001**	0.086
	Fat content	0.013	1, 322	0.909	< 0.001
	GSI/RA	177.794	1, 322	**< 0.001**	0.356
Habitat type	Lean weight	5.627	1, 322	0.018	0.017
	Fat content	1.749	1, 322	0.187	0.005
	GSI/RA	0.053	1, 322	0.819	< 0.001
Population (habitat type)	**Lean weight**	17.371	9, 322	**< 0.001**	0.327
	Fat content	1.913	9, 322	0.049	0.051
	GSI/RA	8.307	9, 322	**< 0.001**	0.188
Sex × population (habitat type)	**Lean weight**	6.671	10, 322	**< 0.001**	0.172
	Fat content	0.996	10, 322	0.446	0.030
	GSI/RA	0.892	10, 322	0.541	0.027
SL × population (habitat type)	**Lean weight**	5.305	10, 322	**< 0.001**	0.141
	Fat content	0.395	10, 322	0.949	0.012
	GSI/RA	1.147	10, 322	0.326	0.034

Table 5.5. *Post-hoc* univariate GLMs on offspring-related life-history trait variation. α-levels have been corrected for multiple comparisons with $\alpha' = 0.017$. Significant effects are highlighted in bold.

Factor	Dependent variable	F	Degrees of freedom	P	Partial η^2
SL	**Fecundity**	53.615	1, 128	**< 0.001**	0.295
	Embryo lean weight	2.955	1, 128	0.088	0.023
	Embryo fat content	0.007	1, 128	0.935	< 0.001
Embryo stage	Fecundity	2.957	1, 128	0.088	0.023
	Embryo lean weight	0.995	1, 128	0.320	0.008
	Embryo fat content	4.026	1, 128	0.047	0.030
Habitat type	Fecundity	0.254	1, 128	0.615	0.002
	Embryo lean weight	7.007	1, 128	**0.009**	0.052
	Embryo fat content	2.006	1, 128	0.159	0.015
Population (habitat type)	**Fecundity**	10.564	9,128	**< 0.001**	0.426
	Embryo lean weight	5.745	9,128	**< 0.001**	0.288
	Embryo fat content	4.934	9,128	**< 0.001**	0.256
SL × population (habitat type)	Fecundity	2.076	10, 128	0.031	0.140
	Embryo lean weight	1.591	10, 128	0.116	0.111
	Embryo fat content	0.729	10, 128	0.696	0.054

Discussion

Most of the phenotypic responses to oil pollution highlighted by our analyses did not follow our *a priori* predictions (Table 5.6). Guppies living in polluted environments were bigger, with rounder and deeper bodies, bigger heads, and increased offspring size compared to those living in non-polluted habitats. Among these, only increased head size points towards direct, negative effects of pollution, while the other trait values suggest that indirect, positive effects might be driving phenotypic responses in these habitats.

Since we were not able to quantify the actual level of pollution experienced by each population, only the presence/absence of visible crude oil in the water, conclusions from our analysis will have to be taken with caution. It is therefore possible that the high levels of variation that we found between populations within the same habitat type, as well as the lack of predicted responses might reflect different pollution levels experienced by each population, and/or a previous history of oil pollution in currently non-polluted environments (Rolshausen et al. 2015).

Alternatively, it has to be noted that these habitats might also experience seasonal variation in their concentrations of crude oil. Guppies were only sampled at the very end of the dry season (January – May). We cannot exclude that, during the wet season (June – December), the concentration of crude oil experienced by some populations might decrease due to the abundant precipitations, thus decreasing the overall strength of selection due to crude oil. Future studies will need to quantify the pollution levels in these environments, as well as the consistency of phenotypic responses across multiple seasons.

Table 5.6. Overview of the effects of oil pollution on guppy (*a*) body-shape and (*b*) life-history traits.

	Traits	'Habitat type'-effect	As predicted? (Y/N)
(*a*) Body shape	Head size	▲ in polluted environments	Y
	Body depth and roundness	Rounder, deeper bodies in polluted environments	N
(*b*) Life history	SL	▲ in polluted environments	N
	Lean weight	No significant effect	-
	Fat content	No significant effect	-
	GSI/RA	No significant effect	N
	Fecundity	No significant effect (▲ in polluted environments IF outliers are not considered)	N
	Embryo weight	▲ in polluted environments	N
	Embryo fat content	No significant effects	-

Body-size variation

In opposition to our *a priori* predictions, guppies living in polluted environments were bigger than in non-polluted ones. This result is slightly puzzling, as it directly contradicts what was previously found in guppies from the Pitch Lake (Santi et al. 2019). Furthermore, reduced body size and hampered growth in response to the presence of oil pollution has been shown in other species of fish, such as *Oreochromis mossambicus* (Saha and Konar 1984a) and rainbow trout *Oncorhynchus mykiss* (Rowe et al. 1983b). Among the multiple factors that could play a role in explaining this surprising pattern in guppies is the presence of parasites. A previous study on guppies in the Pitch Lake showed that this populations lacks gyrodactylid external parasites (Schelkle et al. 2012). Indeed, water pollution has been shown to have a direct toxic effect on *Gyrodactylus turnbulli*, an external parasite of the guppy (Gheorghiu et al. 2006), and even the presence of low concentrations of aluminium and other metals in the water eliminated *G. salaris* infections in the Atlantic salmon *Salmo salar* (Soleng et al. 1999, Poléo et al. 2004). In guppies, larger individuals usually carry a higher parasite load and experience the highest mortality rates (Cable and van Oosterhout 2007, van Oosterhout et al. 2007). This might result in a reduced body size in highly parasitized (i.e., non-polluted) populations.

Similarly, guppies appear to be more tolerant to water pollution than many predator species (Rolshausen et al. 2015). Indeed, in polluted habitats the only other species observed was the killifish *Anablepsoides hartii* (previously known as *Rivulus hartii*; Mohammed et al. 2010, Schelkle et al. 2012, FS pers. obs.), which tends to prey on guppy juveniles (Reznick and Endler 1982). In non-polluted environments on the other hand we observed several potential guppy predators, among which were *Astyanax bimaculatus* (Magurran and Seghers 1990), Guyana leaffish *Polycentrus schomburgkii*, dragonfly larvae (Reznick et al. 2001), and blue heron *Ardea herodias*. In low-predation environments, guppies are usually characterised by increased body size than compared to individuals from high-predation ones (Reznick and Endler 1982). The combination of reduced parasite numbers and low predation could therefore favour increased body size in guppy populations living in polluted environments (Reznick and Endler 1982, Schelkle et al. 2012). Nonetheless, an exception to this pattern is represented by the Pitch Lake, which is usually considered a high-predation environment due to the presence of both Guyana leaffish (Schelkle et al. 2012) and multiple predatory birds (e.g., yellow-billed tern, *Sternula superciliaris*; black skimmer, *Rhyncops niger*, Santi et al. 2019). Indeed, females from the Pitch Lake were the smallest among all polluted habitats (Table D.3).

A possible confounding factor in our analyses could be in water temperature, in particular in the Pitch Lake. There, guppies can be exposed to temperatures up to 41°C (Magurran 2005), close to their critical thermal maximum (Chung 2001). Temperature is known to influence guppy life histories, with fish reared at lower temperatures attaining bigger body size than those reared at higher temperatures (Liley and Seghers 1975), and in fact females (but not males) from the Pitch Lake were smaller than in all but two other populations. While preliminary screening of our data did not show any significant influence of water temperature on guppy phenotypes, we temperature was measured only once for each site, immediately after sampling. Our temperature measures might therefore not have been able to capture the full extent of temperature variation in these environments (both in terms of maximum temperature and daily temperature variation).

Altogether, this suggests that population-specific variation in predation pressure and parasite load, but also thermal stress, might be responsible for the significant variation that we found between populations, independently of habitat type.

Body-shape variation

Alongside increased body size, guppies living in oil-polluted environments were also characterised by increased head size (as predicted) and rounder, deeper bodies (opposite to the predicted pattern) than those living in non-polluted habitats. Increased head size has repeatedly been described for multiple species of fish inhabiting polluted waters. Among these are a previous study on guppies from oil-polluted habitats in Southern Trinidad

(Rolshausen et al. 2015), and tilapia, *Oreochromis* spp. (Sun et al. 2009). Furthermore, increased head size has also been demonstrated in other poeciliid species that have independently colonised environments toxic due to the presence of hydrogen sulphide (e.g., *Poecilia mexicana*, *P. reticulata*, *Gambusia* spp., Tobler and Hastings 2011, Riesch et al. 2016). Among the main effects of oil pollution are respiratory distress (Saha and Konar 1984b), malformations in the gill region (Sun et al. 2009), and increased head size, which is linked to the enlargement of the gills (Haaparanta et al.1997, Fracácio and Verani 2003, Tkatcheva et al. 2004). Larger gills can increase oxygen absorption efficiency in oxygen-depleted environments (Tobler and Hastings 2011), and decrease the uptake of pollutants by thickening the epithelium of the gills and increasing the production of mucus (Fracácio and Verani 2003, Tkatcheva et al. 2004).

Contrary to our predictions, guppies from polluted environments had rounder bodies than those living in non-polluted ones, with this effect being much stronger in females than in males. This is again opposite to what has previously been found in guppies in oil-polluted environments in southern Trinidad (Rolshausen et al. 2015), as well as other poeciliid species from hydrogen sulphide-toxic springs (Riesch et al. 2016). While rounder bodies have a lower body surface-to-volume ratio, which should help decrease the intake of pollutants through the skin (Jahn et al. 1997), multiple environmental factors have been known to influence body shape in guppies, among which are predation (Langerhans and Dewitt 2004) and other habitat features such as canopy cover, flow regime (Hendry et al. 2006) and food availability (Robinson and Wilson 1995). In particular, in low-predation environments, guppies are characterised by shallower and more elongated bodies than in high-predation ones (Langerhans and DeWitt 2004, Burns et al. 2009). Moreover, this effect would be stronger in females than in males, as females become less streamlined due to the distension of the belly towards the latter stages of pregnancy, thus being less able to escape predators (Fleuren et al. 2018). Furthermore, females are preferentially targeted by predators due to their bigger size and higher nutritional value (Pocklington and Dill 1995). However, we also found strong differences between populations regardless of habitat type, again suggesting that population-specific environmental variation, for instance in food availability or in water velocity, could influence this pattern.

Life-history variation

We also found that guppies living in polluted environments were characterised by increased offspring size. While this again contradicts our *a priori* predictions, similar responses have also been found in poeciliids inhabiting hydrogen sulphide-toxic habitats (Riesch et al. 2014). Bigger offspring have lower body-surface-to-volume ratio, which again could reduce the intake of toxins especially during early life-stages, which are especially vulnerable to the negative effects of pollution (Cherr et al. 2017). However, life-history theory predicts increased offspring size both in low-quality environments (Rollinson and Hutchings 2013) and in habitats with higher competition for resources (Bashey 2008), such as low-predation environments, where guppies reach high population densities (Magurran 2005). It remains difficult however to dissect the relative contribution of direct effects of oil pollution and indirect effects due to the absence of predators.

While we did not find a significant effect of habitat type on fecundity, visual inspection of the data (Table D.3) suggested that this lack of response was mainly due to the very high fecundity that we found in population 2. Indeed, after removing population 2 from the analysis, fish living in polluted environments had significantly higher fecundity than those from non-polluted ones. This is again a slightly surprising result, due to the classical life-history trade-off between offspring size and fecundity in poeciliid fishes (Moore et al. 2016). The presence of "super" phenotypes which apparently are opposite to life-history theory has been highlighted before, often in relation to further, previously undetected, selective pressures (Reznick et al. 2000). Similar to our study, Riesch et al. (2014) also found both increased offspring size and increased fecundity in guppies living in sulphur springs in Venezuela. Future studies will need to further investigate these surprising patterns.

A further environmental factor that might have had an effect in our populations was food availability, which we could not quantify during our sampling. The presence of crude oil should decrease food availability as it has negative effects on insect populations and macrophytes (Cushman and Goyert 1984), which are the main components of guppy diet (Zandona et al. 2011). However, the great influx of organic matter via crude oil could potentially also lead to increased food availability (Araújo et al. 2009). Nevertheless, the effects of higher food availability should strengthen, not diminish, the phenotypic responses that we found, in particular with regard to increased body size and offspring size (Grether et al. 2001).

In conclusion, our results paint a complex picture of multiple phenotypic responses to oil pollution in guppies. Guppies apparently only suffered from some negative effects of crude oil (suggested by increased head size and offspring size). In contrast, increased body size in guppies inhabiting polluted environments suggests the presence of indirect, positive effects of oil pollution, potentially in the form of reduced parasite load and predation pressure. Nevertheless, future studies should further characterise the extent of ecological variation in oil-polluted aquatic habitats to be able to identify the other selective forces (besides oil toxicity) that might act upon organisms in these environments and their relative importance in driving phenotypic differentiation.

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Chapter 6: General discussion

Overview of the main results

How predictable are the effects of environmental gradients on livebearing fishes? Across all my data chapters, I found that, while some phenotypic responses to environmental gradients were predictable, many others were not predictable, and the degree of predictability varied greatly depending on both the phenotypic traits and the gradients considered.

In **Chapter 2**, I focused on diversification along environmental gradients on a relatively small spatial scale (i.e., 0.06 degrees latitude and 0.25 degrees longitude). My analyses on the relative contribution of multiple environmental gradients in driving phenotypic differentiation in *P. januarius* populations from coastal lagoons in Brazil highlighted the importance of multifarious selection in this species. Surprisingly, differences in pH and oxygen availability were found to be more important for phenotypic diversity than difference in salinity and predation pressure, despite the latter two having previously been proposed to be the main selective pressures in this system.

In **Chapter 3**, I then investigated diversification on a much greater spatial scale, spanning three countries (i.e., 11.03 degrees latitude and 24.14 degrees longitude). Using population genetics tools, my co-authors and I confirmed a single introduction of invasive mosquitofish *G. holbrooki* in Europe. Analyses on life-history and body-shape diversification across France, Italy, and Spain showed that mosquitofish underwent phenotypic differentiation across a latitudinal gradient during their range expansion and are now characterised by increased body size in northern populations. However, phenotypic responses were also strongly influenced by climate-independent environmental variation (in particular, again, oxygen availability) and temporal variation. I then demonstrated for the first time—using a common garden experiment—that phenotypic differences are mainly due to (adaptive) phenotypic plasticity, with rapid evolutionary change playing a much smaller role.

In **Chapter 4**, still focusing on invasive mosquitofish, but now also including populations from a second species (*G. affinis*) across China, I analysed both the direct and indirect effects of climate on geographical and temporal variation of multiple paternity in invasive mosquitofish. I found higher levels of multiple paternity in northern populations of both species. However, the effects of climate were mostly indirect, mediated by life-history differences.

Lastly, in **Chapter 5**, I again focused back on a relatively small spatial scale (0.52 degrees latitude and 0.39 degrees longitude) to investigate whether the presence of a strong selective pressure can increase predictability of patterns of phenotypic diversification. For this purpose, I analysed guppies (*P. reticulata*) subject to oil pollution in Trinidad. Once more, I found a combination of predictable and unpredictable responses, most of which were arguably driven by a combination of the negative effects of oil pollution and the positive effects of reduced predation pressure and parasite load in polluted environments.

How consistent and predictable were the phenotypic effects of environmental gradients?

Across my data chapters, I analysed predictable phenotypic effects of a wide range of environmental gradients in four species of livebearing fish over widely different geographical scales. Nevertheless, multiple chapters investigated the effects of the same (or similar) environmental gradient, and it is therefore possible to draw some conclusions on the consistency of phenotypic response across systems.

In particular, I analysed the effects of temperature variation in Chapters 2, 3, and 4, even though each chapter focused on different aspects of such variation. In Chapter 2, I used water temperature measured during sampling in order to describe (mainly) seasonal differences between dry and wet seasons (i.e., the Brazilian winter and summer, respectively). In Chapter 3, I considered weather data averaged over 120 days preceding the sample, in order to account for both seasonal (as some fish were sampled towards the beginning of the reproductive season and others towards the end) and yearly (as fish were sampled in both 2013 and 2017) temperature differences over a latitudinal gradient. Lastly, in Chapter 4, I focused on climate differences experienced by mosquitofish populations—again over a large-scale latitudinal gradient—and used long-term climate data, averaged over a period of 30 years (1970-2000).

Despite these differences, environmental temperatures affected fish phenotypes in all three studies. In chapter 2, *P. januarius* were characterised by reduced fecundity, superfetation, and reproductive allocation (in the case of females), as well as reduced GSI (in the case of males) in higher temperatures. In Chapter 3, temperature affected mosquitofish body size, as *G. holbrooki* were bigger in colder environments, with males also being characterised by higher GSI (contrary to my findings for *P. januarius* in Chapter 2). My results from Chapter 4 broadly confirmed some previous studies, as increased temperatures over a north-south gradient had a weak, negative effect on female SL, fecundity, and RA, as well as male GSI, which in turn resulted in increased multiple paternity in northern populations. In Chapter 4, however, temperature variation was also described by an inland-coastal geographical gradient (with increased annual temperature differences in inland sites), which had a similar effect on the same life-history traits, as well as a negative effect on female lean weight.

While these responses varied between species, they were consistently different from what was previously found in a study that experimentally manipulated water temperature of *G. affinis* in the laboratory (Vondraceck et al. 1988). In their study, Vondraceck et al. (1988) found that fish raised at 30°C had faster growth rate, higher fecundity and offspring weight than fish raised at both 25°C and 20°C, while I found reduced reproductive output and reduced body size in higher temperatures.

Several, non-mutually exclusive explanations can be invoked to understand the overall negative effects of higher temperatures on fishes in my analyses, but also the differences between the different study systems. Firstly, higher temperatures usually cause increased metabolism in ectotherms (Johnston and Dunn 1987), thus making food availability a potential limiting factor for fish growth and reproduction (Reznick 1983). In guppies, for instance, fecundity and reproductive allocation were found to be lower during the wet season (similarly to what I found in *P. januarius*), and these patterns were consistent with a reduced food availability (Reznick 1989). Secondly, experimental manipulation of water temperature in the laboratory does not take into account the daily and yearly temperature variation experienced by fish in the field. In *Gambusia*, for instance, overwinter mortality, especially in northern populations, can reach 99% (Chen et al. 2018), and bigger individuals likely have increased survival in cold environments (Riesch et al. 2018). It is therefore possible that the survival benefits of increased body size in cold habitats might overshadow any growth increases in warmer ones. Lastly, I used different temperature data across my studies. While water temperature measurements describe the temperature actually experienced by the fish better than long-term climate data, they cannot reflect the entirety of temperature variation over a long period of time. At the same time, however, using long-term climate data across multiple studies can have limitations due to the spatial resolution of the data itself (for instance, in Chapter 2), or due to their inability to describe yearly or seasonal variation (as in the case of Chapter 4). Future improvements and standardisation of weather and climate data will greatly help to fully understand how consistent phenotypic responses are across different study systems.

Multifarious selection

In natural populations, organisms are usually subject to multifarious selection, a complex mix of selective pressures from multiple, potentially interacting, environmental factors (Riesch et al. 2020). The importance of multifarious selection for phenotypic differentiation (but also speciation, e.g., Nosil 2012) has been fully recognised in recent years, with more and more examples in the published literature (e.g., Johnson and Kliman 2002, Pfrender 2012, Egea-Serrano et al. 2014), including studies on livebearing fishes (e.g., Riesch et al. 2020). Furthermore, several studies on phenotypic divergence across environmental gradients (e.g., Langerhans 2018, Rius et al. 2019, Riesch et al. 2020) highlighted how the patterns observed might be influence also by the presence of correlations between different suites of phenotypic traits (i.e., covariation). For example, in poeciliids, body-shape variation appears to be driven by variation in life-history traits (Johnson and Bagley 2011; Riesch et al. 2020), and sometimes also vice versa (Riesch et al. 2020); nevertheless such correlations might potentially arise between any set of phenotypic traits.

Across all chapters, my results highlighted how indeed the patterns of phenotypic differentiation were influenced simultaneously by multiple environmental gradients (multifarious selection), or by covariation between different phenotypic traits (for example in Chapter 4, where multiple paternity differences were mostly driven by life-history variation), even when focusing on the most striking (and potentially important) environmental difference between two sets of habitats.

The importance of multifarious selection was especially evident in Chapters 2 and 5, where I focused on two study systems that had previously been described to be dominated by a single, strong selective pressure (Chapter 2, predation risk: Neves and Monteiro 2003, Gomes-Jr and Monteiro 2007, Gomes and Monteiro 2008, Araújo et al. 2009, Rius et al. 2019; Chapter 5, oil pollution: Rolshausen et al. 2015, Hamilton et al. 2017). It could be expected that focusing on a strong selective pressure that imposes immediate survival costs to organisms might increase predictability of phenotypic responses, as these effects might override weaker concomitant selective forces (Moore et al. 2016). Nevertheless, Langerhans (2018) noted that differences in predation risk only explain less than half of the total phenotypic variation of Bahamian mosquitofish *G. hubbsi*, even though multiple responses were predictable across several trait suites.

My analyses appeared to corroborate this finding. While I found that the responses to predation were generally in the predicted direction in Chapter 2, their relative importance was surprisingly low, with oxygen availability and pH having stronger effects (which are discussed below). My results from Chapter 5 appear to be even more puzzling, as the presence of oil pollution (which has been shown to have extremely toxic effects in several fish species; Hamilton et al. 2017) did not affect adult lean weight, fat content, and GSI/RA, and its effect on SL, fecundity and offspring weight (all of which were increased in polluted habitats) were opposite to what I predicted *a priori*. A possible explanation for this can be found in the "enemy release hypothesis", first introduced to explain the invasion success of alien species as a result of the lack of their "enemies" (predators and parasites) in their invasive range (Colautti et al. 2004). Indeed, it appears as if, at least in the case of guppies, degraded habitats act as "refuges" from even stronger selective pressures such as those derived from predators or parasites (Schelkle et al. 2012).

Overall, these results support the notion that the presence of an apparently strong selective pressure might not necessarily increase predictability of phenotypic differentiation. To the contrary, they paint an extremely complex picture, where it is necessary to consider both multiple environmental factors as well as their effects on a community level.

Effects of oxygen availability and pH

Among the most surprising results of Chapters 2 and 3 were the strong effects that oxygen availability had on phenotypic differentiation in both *P. januarius* and *G. holbrooki.* In Chapter 2, *P. januarius* living in high-oxygen conditions had reduced fecundity, superfetation, MI, and RA (in females), as well as GSI (in males). Meanwhile, in Chapter 3, meanwhile, *G. holbrooki* living in low-oxygen environments were characterised by increased body size and reduced offspring size, whereas the effects of oxygen availability on fecundity and RA were opposite between the two sampling years, and there was no effect of oxygen levels on male GSI.

This effect is especially puzzling given that most research efforts so far have concentrated on how hypoxia influences life-history, morphology and behaviour of fishes (Chapman 2015). However, in the case of the studies presented in Chapters 2 and 3, these effects do not appear to be relevant as most (in the case of *G. holbrooki*) or all (in the case of *P. januarius*) populations were characterised by oxygen concentrations above those usually considered hypoxic (2mg O_2/L; Chapman 2015).

Among the mechanisms that might be invoked to help explain these results is the presence of oxidative stress, which is considered to be a common constraint of life-history evolution (Dowling and Simmons 2009). Oxidative stress is caused by reactive oxygen species (ROS), which are a common byproduct of oxygen metabolism, especially in hyperoxic (but also in hypoxic) conditions (Lushchak and Banyukova 2006). One of its main consequences is that it increases the costs of reproduction in multiple organisms. For example, in both *D. melanogaster* (Wang et al. 2001) and zebra finches, *Taeniopygia guttata* (Alonso-Alvarez et al. 2004), increased reproductive effort resulted in increases in oxidative stress. However, in the Nile tilapia, *Oreochromis niloticus*, females raised at medium or low oxygen levels had lower GSI and fecundity than those raised in high-oxygen conditions (Kolding et al. 2008). These results corroborate what previously found in *G. hubbsi*, where fecundity appeared to increase together with oxygen availability (Riesch et al. 2015), but they are apparently opposite to what I found in my research chapters.

It is therefore possible that my results reflect mostly indirect effects of oxygen. Different species have different optima of oxygen availability, and the distribution of some fish species can be limited by the amount of dissolved oxygen (e.g., *Hoplias malabricus*, Petry et al. 2013; lake trout, *Salvelinus namaycush*, Sellers et al. 1998; Nile perch, *Lates niloticus*, Goudswaard et al. 2011), thus impacting other environmental factors, such as predation risk, competition, or food availability, which, in turn, can then affect life-history differentiation. Indeed, in the case of Chapter 2, the main predator species of *P. januarius*, *Hoplias malabricus*, is limited to highly oxygenated environments, and oxygen variation can therefore reflect more nuanced differences in predation risk (Petry et al. 2013). Moreover, previous studies on *G. holbrooki* from rice fields in Portugal showed a negative correlation between oxygen levels and the number of invertebrates predated upon by the fish (Cabral et al. 1998), and, consequently, a negative correlation with fecundity (Cabral and Marques 1999).

Similar considerations can be made in the case of pH variation. In high-pH conditions, *P. januarius* were characterised by increased body size and reproductive investment compared to low-pH conditions (while in Chapter 3 pH did not significantly affect *G. holbrooki* phenotypes). Once more, these results did not align with my *a priori* predictions. pH variation is known to cause physiological stress in fishes, in particular during early life-stages (Crespel et al. 2017), and increased pH should, if anything, decrease body size and reproductive investment (Brown-Peterson and Peterson 1990, Riesch et al. 2015, Jourdan et al. 2016). It remains extremely complicated to interpret these results, given the lack of studies that specifically investigated the effects of pH on livebearing fish phenotypes. Nevertheless it is possible that, once more, species-specific pH tolerance might play a role in these responses. Indeed, low pH has been shown to decrease reproduction in other species of fish (e.g., flagfish, *Jordanella floridae*, Ruby et al. 1977; *Cyprinodon nevadensis nevadensis*, Lee and Gerking 1980; *Xiphophorus helleri*, Rubin 1985).

In both cases, it appears that these environmental gradients play underestimated roles in shaping phenotypic differentiation of livebearing fishes, and therefore warrant further research, both with regards to their direct and indirect effects.

Temporal variation, phenotypic plasticity, and rapid evolution

One of the general trends that was evident across my data chapters was the influence of temporal variation on most phenotypic responses considered, as several responses to environmental gradients disappeared or even changed direction between seasons or sampling years. This effect was especially evident in Chapter 3 (as discussed in Appendix B5), but was also present in Chapters 2 (as previously discussed in relation to the effect of temperature differences) and 4, where I did not only find variation in multiple paternity levels during the reproductive season, but also between sampling years, as fish from the Beihai population had consistently higher multiple paternity levels than those from Ankang in the analysis of temporal variation of multiple paternity, but the opposite was true for my analysis of geographical variation, whose fish were sampled the previous year. In all chapters, I controlled for seasonal or yearly variation mainly using different climatic and temperature measures, nevertheless it was impossible to exclude that other environmental factors may have varied between samplings. In particular, one factor that was not quantified during sampling was population density, whose influence on livebearing fish phenotypes is well-established in the published literature (e.g., Bisazza and Marin 1995, Smith and Sargent 2006, Reznick et al. 2002, 2012, Bassar et al. 2013). Regardless of the mechanism, my results point towards the presence of high levels of phenotypic plasticity as a strategy to cope with highly variable or fast-changing environmental conditions (Ghalambor et al. 2007), as demonstrated in several species of fish (Belk 1995, Pampoulie et al. 2000, Baker et al. 2015).

Phenotypic plasticity, or the ability of a single genotype to produce different phenotypes depending on environmental conditions (West-Eberhard 2003), allows organisms to immediately respond to changes in their environment (West-Eberhard 2003). At the same time, previous studies have shown that livebearing fishes are able to quickly adapt to local environmental conditions such as in high vs. low-predation guppies (Reznick et al. 1996), including fine-scale local adaptation along gradual variation in predation risk (Torres-Dowdall et al. 2012). Moreover, local life-history adaptation to differences in water temperature was found between four population of recently translocated *G. affinis* in the U.S.A. within 55-58 years of introduction (Stockwell and Weeks 1999).

I specifically investigated the relative contributions of phenotypic plasticity and rapid evolutionary change in the phenotypic differentiation on invasive European *G. holbrooki* in Chapter 3. My results demonstrated that most phenotypic variation was driven by (adaptive) phenotypic plasticity, with rapid evolution playing a much-reduced role, as only male body size and embryo fat content showed moderate heritability across generations.

It remains difficult to quantify the relative contribution of plasticity and rapid evolution on phenotypic differentiation, with common-garden or experimental evolution studies remaining our best tools to investigate these processes (Merilä and Hendry 2014). Nevertheless, comprehending the complex interplay between these two processes remains important, in particular in order to predict species invasiveness (Hendry 2015), but also to understand which aspects of environmental variation will promote evolutionary changes. Moreover, long-term studies on phenotypic variation that concentrate on the repeatability of phenotypic responses between different seasons and years, will also help to increase our understanding of these processes.

Future environmental challenges

As I stated in the introduction, being able to predict how organisms react to environmental variation is extremely relevant in light of the environmental challenges that organisms are facing now and are predicted to face in the near future (Sarrazin and Lecomte 2016). I specifically focused on anthropic impacts on ecosystems in Chapters 3, 4 (which dealt with the invasiveness of eastern and western mosquitofish) and 5 (which analysed the effects of anthropogenic oil pollution on guppies). Humans are one of the main sources of environmental change worldwide. Human activities often have widespread negative effects for organisms but, simultaneously, can be a source of evolutionary change (Palumbi 2001).

Across all chapters, a common factor in the success of *P. januarius*, mosquitofish, and guppies was their ability to cope with a wide range of environmental conditions (Evans et al. 2011), allowing them to thrive in otherwise degraded habitats (e.g., Araújo et al. 2009, Gomes-Silva et al. 2019). In mosquitofish, my results from Chapter 3 showed how high levels of phenotypic plasticity allowed them to react to novel environmental conditions during their extremely successful European invasion and subsequent range expansion over the last 100 years. Furthermore, in Chapter 4 I highlighted how life-history trait variation across the ranges of both *G. holbrooki* and *G. affinis* resulted in increased levels of multiple paternity in populations of both species living at higher latitudes. The northern expansion of mosquitofish appears to be limited by colder temperatures (Otto 1973). However, higher levels of multiple paternity imply that offspring in northern locations have higher levels of genetic variation and/or genetic quality (Jennions and Petrie 2000), both of which are adaptive in harsh environments (Botero and Rubenstein 2012). Therefore, my results show that mosquitofish are potentially perfectly primed to expand their range following the temperature increases predicted in the next few decades due to global warming (Carboni et al. 2017), thus increasing their range and their negative effects on native fauna (Kottelat and Whitten 1989).

One of the main consequences of pollution, and in particular of oil pollution, is a drastic reduction in biodiversity in the impacted habitats (Cohen et al. 1993, Edinger et al. 1998). Guppies, thanks to their ability to tolerate even strongly polluted habitats, have been able to colonise several polluted streams throughout southern Trinidad (Rolshausen et al. 2015), as well as other degraded environments across South America (e.g., Araújo et al. 2009, Casatti et al. 2009, Gomes-Silva et al. 2019), where they are the dominant species. In Chapter 5, I analysed phenotypic responses to oil pollution in several Trinidadian guppy populations and, once more, my results were surprising, consisting in both (apparently) negative effects of oil pollution and (apparently) positive effects, likely as a consequence of reduced predation and/or parasitism. A previous study on guppies living in oil-polluted environments failed to uncover any evidence of local adaptation to pollution (Rolshausen et al. 2015), contrary to what had been demonstrated in several other species of fish (Ownby et al. 2002, Meyer and Di Giulio 2003, Williams and Oleksiak 2008).

My results show how the reduction in biodiversity might actually favour some, highly generalist species, even in the absence of local adaptation to a specific environmental stressor (Devictor et al. 2008). This is important knowledge as more and more habitats become polluted due to human activities. Since most invasive species are generalists (e.g., Marvier et al. 2004), able to cope with a wide range of environmental conditions (Devin and Beisel 2007), and characterised by high levels of phenotypic plasticity (Davidson et al. 2011; Chapter 4), it is therefore likely that increased habitat destruction and pollution might favour the range expansion of these species, further exacerbating the negative effects on native fauna and biodiversity loss.

Conclusions, open questions and future directions

The study of predictable phenotypic responses to environmental gradients has made great strides over the last few years (Lässig 2017). Yet, due to the ever-increasing speed of environmental change which is caused especially by human impacts (Sarrazin and Lecomte 2016), we still need to gain further understanding. As outlined in this discussion as well as in the previous chapters, multiple research questions remain unanswered, and several others have emerged based on my results. Answering these questions will be of the outmost importance in the next few years, as organisms continue to face unprecedented environmental challenges of habitat destruction, climate change, or the introduction of alien species. These studies will be relevant not only from a theoretical point of view, but also from a practical one, for example informing policy makers for future development plans and conservation efforts (Stockwell et al. 2003), eventually transitioning from being able to predict evolutionary outcomes, to be able to ‘control’ them (Lässig et al. 2017).

Across my data chapters, I investigated how several environmental factors affected phenotypic differentiation of three different species of livebearing fishes at widely different geographical scales. My results showed that, overall, it is possible to predict phenotypic responses, and that the effects of some environmental gradients (e.g., temperature) were somewhat consistent across systems. However, the degree of predictability was greatly diminished by the combined effect of multiple environmental gradients. Future studies will need to further concentrate on the effects of multifarious selection on phenotypic differentiation in order to better understand why phenotypic responses to certain environmental factors appear to be more predictable than others, as well as what might drive “unique features of differentiation” (Langherhans and DeWitt 2004) between populations apparently subject to the same environmental gradients.

My analyses further highlighted how some, usually overlooked environmental gradients had surprising important effects on phenotypes, independently of interactions between different environmental gradients. Even employing a multivariate approach, our ability to formulate accurate *a priori* predictions hinges on a correct understanding of the effects of each individual environmental gradient. In particular, as the effects of more and more environmental gradients become evident, future studies will need to investigate how exactly these as yet understudied environmental variables (e.g., oxygen availability and pH) drive phenotypic diversification, for instance through experimental manipulation.

While I mainly focused on phenotypic differentiation, I specifically analysed the mechanisms behind these phenotypic responses—namely phenotypic plasticity and rapid evolution—in Chapter 3. While the published literature showcases multiple instances of rapid adaptation to changing environmental conditions in livebearing fishes (Reznick et al. 1990, Stockwell and Weeks 1999, Plath et al. 2007), my results showed that phenotypic variation in invasive *G. holbrooki* was mainly due to plasticity, with heritable, genetic changes playing only a much smaller role. Future studies will need to further explore the differences between plastic responses and evolved ones, focusing in particular on which environmental factors do actually promote rapid evolution, and which ones favour instead adaptive phenotypic plasticity. In both cases, comparative transcriptomics and genomics studies will likely be needed in order to fully understand the complex interplay between phenotypic plasticity and evolutionary diversification by identifying the regions of genome that are implicated with evolutionary divergence, as well as investigating how environmental variation influences the expression and regulation of particular genes.

To conclude, our ability of predicting phenotypic responses to environmental gradients—though much improved recently—remains in many ways somewhat limited. My thesis work helped to shed some light on some of these processes, as well as highlight topics that warrant further research. This brings us a small step closer to being able to accurately answer the question “as habitats change, will organisms be able to adapt?”.

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Appendix A: Supplementary material for Chapter 2 – Phenotypic differentiation in a heterogeneous environment: morphological and life-history responses to ecological gradients in a livebearing fish

Geometric morphometrics

We took standardized lateral photographs of each fish using a Canon EOS 1200D digital camera (Canon Inc., Tokyo, Japan) with a fixed 60mm macro lens mounted on a copy stand. All pictures were collated together into a TPS file using tpsUtil32 (Rohlf 2016a), and, using tpsDig232 software (Rohlf 2016b), we carefully positioned the following 15 landmarks on each picture of a fish: (1) tip of the upper jaw; (2) anterior and (3) posterior insertions of the dorsal fin; (4) dorsal and (5) ventral insertions of the caudal fin; (6) posterior and (7) anterior insertions of the anal fin; (8) anterior junction of the pelvic fin; (9) bottom of the head where the operculum breaks away from the body outline; (10) dorsal end of the operculum; (11) dorsal and (12) ventral insertions of the pectoral fin; (13) centre of the eye; (14) anterior and (15) posterior edge of the orbit (see Riesch et al. 2016; Fig. A.1). To account for bent specimens due to preservation, we used the "unbend specimen" function in tpsUtil32. We used the landmark at the tip of the upper jaw, as well as 3 temporary landmarks (middle of the caudal peduncle and 2 additional landmarks along the lateral line). The temporary landmarks were then removed from the final analysis. In total, 83 fish were unbent (2 from Bezerra, 36 from Catingosa, 24 from Maria Menina and 21 from Pitanga). Finally, we excluded from the analysis 3 individuals which remained outliers even after the unbending (all from Catingosa). The coordinates of the landmarks were analysed using the program tpsRelw32 (Rohlf 2016c). This program first aligns the specimens using least square superimposition to remove effects of rotation, translation and scale (Rohlf 1999), and then performs a relative warps analysis (Zelditch et al. 2012). The first two relative warps extracted accounted for more than 90.24% of the cumulative variance in the sample and were used as shape variables for subsequent analyses (Table A.1). As part of the relative warps analysis, the tpsRelw32 program automatically calculates also the centroid size of each individual, as the square root of the sum of the squared distances of each landmark from their centroid. We then used 'centroid size' as a covariate in subsequent analyses to account for differences in body size between individuals.

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Supplementary tables and figures

Table A.1. Mean ± SE of female life-history traits and life-history proxies.

Lagoon	Date	*N*	SL [mm]	Lean weight[1] [mg]	Fat content [%]	Fecundity[1]	Est. offspring size at birth [mg]	MI	Superfetation[1]	Embryo fat content [%]	RA [%]
Bezerra	Jan-12	20	27.59 ± 0.95	124.40 ± 3.84	3.22 ± 0.60	29.11 ± 2.03	1.95 ± 0.12	102.52 ± 42.97	7.13 ± 0.32	11.12 ± 1.91	4.14 ± 0.61
	Jul-12	20	27.88 ± 0.45	116.12 ± 3.79	1.58 ± 0.36	16.97 ± 2.01	1.96 ± 0.14	44.10 ± 15.02	6.54 ± 0.32	5.81 ± 0.64	3.67 ± 0.32
	Total	40	27.73 ± 0.52	120.26 ± 2.84	2.40 ± 0.37	23.04 ± 1.51	1.96 ± 0.09	73.31 ± 22.95	6.84 ± 0.24	8.47 ± 1.08	3.90 ± 0.34
Pitanga	Jul-11	20	32.14 ± 0.66	138.45 ± 3.64	4.44 ± 0.70	39.30 ± 1.93	2.53 ± 0.16	57.98 ± 12.71	7.96 ± 0.31	2.53 ± 0.46	9.10 ± 0.56
	Jan-12	20	34.02 ± 0.49	134.61 ± 3.85	1.97 ± 0.27	34.82 ± 2.04	2.31 ± 0.16	102.17 ± 36.571	6.69 ± 0.32	3.65 ± 0.30	7.32 ± 0.61
	Total	40	33.08 ± 0.43	136.53 ± 2.73	3.20 ± 0.42	37.06 ± 1.45	2.42 ± 0.11	80.08 ± 19.43	7.33 ± 0.23	3.09 ± 0.29	8.21 ± 0.43
Maria Menina	Jul-11	20	31.28 ± 1.00	162.78 ± 3.60	2.87 ± 0.35	19.58 ± 1.91	3.69 ± 0.19	571.60 ± 144.48	5.55 ± 0.30	5.68 ± 1.05	4.25 ± 0.86
	Jan-12	20	32.16 ± 0.52	139.52 ± 3.64	2.47 ± 0.18	20.01 ± 1.93	2.73 ± 0.18	298.27 ± 165.56	6.86 ± 0.31	4.23 ± 0.29	5.56 ± 0.39
	Jul-12	20	31.70 ± 0.37	148.51 ± 3.62	4.77 ± 0.48	10.71 ± 1.92	2.82 ± 0.16	104.02 ± 27.26	5.93 ± 0.30	6.07 ± 0.73	2.49 ± 0.25
	Total	60	31.71 ± 0.39	150.27 ± 2.12	3.37 ± 0.24	16.77 ± 1.12	3.08 ± 0.12	324.63 ± 76.72	6.10 ± 0.18	5.33 ± 0.44	4.10 ± 0.36
Catingosa	Jul-11	20	31.64 ± 0.31	126.96 ± 3.62	1.82 ± 0.33	21.53 ± 1.92	2.62 ± 0.10	110.24 ± 24.59	7.35 ±0.30	2.97 ± 0.34	6.94 ± 0.40
	Jan-12	20	29.34 ± 0.63	121.27 ± 3.64	2.38 ± 0.23	17.36 ± 1.93	2.38 ± 0.12	64.23 ± 31.15	5.59 ± 0.31	5.20 ± 0.72	3.69 ± 0.39
	Jul-12	20	29.67 ± 0.33	134.23 ± 2.62	4.14 ± 0.30	18.17 ± 1.92	2.04 ± 0.13	54.22 ± 26.32	6.85 ± 0.30	6.88 ± 0.83	3.93 ± 0.46
	Total	60	30.22 ± 0.29	127.49 ± 2.09	2.78 ± 0.21	19.02 ± 1.11	2.34 ± 0.07	76.23 ± 15.92	6.60 ± 0.18	5.02 ± 0.43	4.85 ± 0.31

[1] estimated marginal means for SL=30.74 mm.

Table A.2. Mean ± SE of male life-history traits and life-history proxies.

Lagoon	Date	*N*	SL [mm]	Lean weight[1] [mg]	Fat content [%]	GSI [%]
Bezerra	Jan-12	3	17.77 ± 0.44	22.42 ± 1.43	1.45 ± 1.45	2.48 ± 0.22
	Jul-12	3	17.90 ± 0.50	22.55 ± 1.42	2.90 ± 1.46	1.03 ± 0.18
	Total	6	17.83 ± 0.30	22.48 ± 1.02	2.18 ± 0.97	1.76 ± 0.35
Pitanga	Jul-11	6	17.77 ± 0.87	27.92 ± 1.03	2.30 ± 1.05	3.31 ± 0.25
	Jan-12	4	19.30 ± 0.59	27.94 ± 1.24	1.39 ± 0.80	2.11 ± 0.28
	Total	10	18.38 ± 0.60	27.93 ± 0.79	1.94 ± 0.69	2.83 ± 0.27
Maria Menina	Jul-11	10	19.07 ± 0.31	30.26 ± 0.79	2.04 ± 1.11	2.02 ± 0.11
	Jan-12	4	17.88 ± 0.40	26.90 ± 1.24	4.18 ± 1.87	2.70 ± 0.19
	Jul-12	4	19.65 ± 0.38	29.79 ± 1.27	2.25 ± 1.37	1.69 ± 0.16
	Total	18	18.93 ± 0.25	28.98 ± 0.64	2.56 ± 0.79	2.10 ± 0.11
Catingosa	Jul-11	-	-	-	-	-
	Jan-12	-	-	-	-	-
	Jul-12	10	18.40 ± 0.34	26.35 ± 0.77	3.39 ± 1.08	1.66 ± 0.11
	Total	10	18.40 ± 0.34	26.35 ± 0.77	3.39 ± 1.08	1.66 ± 0.11

[1] estimated marginal means for SL=18.54 mm

Table A.3. Environmental parameters measured at each sampling event.

Lagoon	Date	Predation	Salinity [ppt]	Water Temperature [ºC]	Dissolved oxygen [mg/L]	Chlorophyll *a* [μg/L]	pH
Bezerra	Jan-12	High	1.8	28.1	6.62	75.43	6.40
	Jul-12	High	2.0	24.9	8.41	3.73	6.20
Pitanga	Jul-11	High	19.3	21.7	5.37	5.17	6.43
	Jan-12	High	0.2	27.2	6.35	21.78	7.20
Maria Menina	Jul-11	Low	36.2	24.9	5.81	2.01	7.35
	Jan-12	Low	29.0	27.2	5.01	6.42	7.83
	Jul-12	Low	32.1	22.7	9.27	1.33	7.70
Catingosa	Jul-11	Low	22.6	24.7	6.55	1.87	7.82
	Jan-12	Low	17.3	30.3	6.51	11.35	7.89
	Jul-12	Low	15.5	25.9	8.02	2.16	8.20

Table A.4. Relative Warps (RW) and % of variance explained. Only RW1 and RW2 were used in the analyses.

RW	Eigenvalues	% Variance explained	Cumulative % var. explained
1	1.668	87.24	87.24
2	0.310	3.01	90.24
3	0.251	1.98	92.22
4	0.222	1.54	93.76
5	0.189	1.12	94.89
6	0.176	0.97	95.86
7	0.167	0.88	96.74
8	0.134	0.56	97.30
9	0.113	0.40	97.70
10	0.105	0.34	98.04
11	0.098	0.30	98.34
12	0.095	0.28	98.62
13	0.091	0.26	98.89
14	0.082	0.21	99.10
15	0.075	0.18	99.27
16	0.068	0.15	99.42
17	0.065	0.13	99.55
18	0.058	0.11	99.65
19	0.056	0.10	99.75
20	0.046	0.07	99.82
21	0.045	0.06	99.88
22	0.035	0.04	99.92
23	0.035	0.04	99.96
24	0.030	0.03	99.98
25	0.018	0.01	99.99
26	0.015	0.01	100.00

Table A.5. *Post-hoc* ANCOVAs on variation in (*a*) body shape, as well as (*b*) female and (*c*) male life-history traits and proxies. Alpha-levels were corrected for multiple comparisons with (*a*) $\alpha' = 0.025$, (*b*) $\alpha' = 0.006$, and (*c*) $\alpha' = 0.017$. Significant effects are highlighted in bold.

	Factor	Dependent Variable	*F*	Degrees of freedom	*P*
(*a*) Body shape	Sex	**RW1**	122.296	1, 232	**< 0.001**
		RW2	15.462	1, 232	**< 0.001**
	Centroid size	**RW1**	18.746	1, 232	**< 0.001**
		RW2	15.958	1, 232	**< 0.001**
	Lagoon	**RW1**	7.014	3, 232	**< 0.001**
		RW2	2.401	3, 232	0.068
	Date(lagoon)	RW1	1.397	6, 232	0.217
		RW2	3.608	6, 232	**0.002**
	Sex × lagoon	**RW1**	6.867	3, 232	**< 0.001**
		RW2	3.498	3, 232	**0.016**
	Sex × date(lagoon)	**RW1**	3.645	4, 232	**0.007**
		RW2	1.174	4, 232	0.323
	Centroid size × lagoon	**RW1**	6.803	3, 232	**< 0.001**
		RW2	2.231	3, 232	0.085
(*b*) Female life-history traits	SL	**Lean weight**	729.595	1, 180	**< 0.001**
		Fat content	3.177	1, 180	0.076
		Fecundity	89.064	1, 180	**< 0.001**
		Est. offspring size at birth	0.125	1, 180	0.724
		MI	1.285	1, 180	0.258
		Superfetation	22.506	1, 180	**< 0.001**
		Embryo fat content	0.028	1, 180	0.866
		RA	1.479	1, 180	0.226
	Lagoon	**Lean weight**	40.733	3, 180	**< 0.001**
		Fat content	1.825	3, 180	0.144
		Fecundity	28.927	3, 180	**< 0.001**
		Est. offspring size at birth	11.384	3, 180	**< 0.001**
		MI	6.326	3, 180	**< 0.001**
		Superfetation	7.703	3, 180	**< 0.001**
		Embryo fat content	5.784	3, 180	**0.001**
		RA	19.274	3, 180	**< 0.001**
	Date(lagoon)	**Lean weight**	6.475	6, 180	**< 0.001**
		Fat content	4.595	6, 180	**< 0.001**
		Fecundity	13.464	6, 180	**< 0.001**
		Est. offspring size at birth	4.197	6, 180	**0.001**
		MI	3.725	6, 180	**0.002**
		Superfetation	9.706	6, 180	**< 0.001**
		Embryo fat content	2.292	6, 180	0.037
		RA	8.845	6, 180	**< 0.001**
	SL × lagoon	Lean weight	1.916	3, 180	0.129
		Fat content	1.539	3, 180	0.206
		Fecundity	0.851	3, 180	0.468
		Est. offspring size at birth	0.790	3, 180	0.501
		MI	0.165	3, 180	0.920
		Superfetation	2.970	3, 180	0.033
		Embryo fat content	3.328	3, 180	0.021
		RA	0.616	3, 180	0.605
	SL × date(lagoon)	**Lean weight**	2.374	6, 180	0.031
		Fat content	0.969	6, 180	0.448
		Fecundity	4.319	6, 180	**< 0.001**
		Est. offspring size at birth	0.986	6, 180	0.436
		MI	1.071	6, 180	0.382
		Superfetation	4.805	6, 180	**<0.001**
		Embryo fat content	2.532	6, 180	0.022
		RA	3.756	6, 180	**0.002**

Table A.5 continued.

(*c*) Male life-history traits	SL	**Lean weight**	110.619	1, 32	**< 0.001**
		Fat content	0.720	1, 32	0.402
		GSI	1.866	1, 32	0.181
	Lagoon	**Lean weight**	6.820	3, 32	**0.001**
		Fat content	0.995	3, 32	0.408
		GSI	10.030	3, 32	**< 0.001**
	Date(lagoon)	Lean weight	0.745	4, 32	0.569
		Fat content	0.825	4, 32	0.519
		GSI	11.097	4, 32	**< 0.001**
	SL × lagoon	Lean weight	0.574	3, 32	0.636
		Fat content	6.488	3, 32	**0.001**
		GSI	1.741	3, 32	0.178

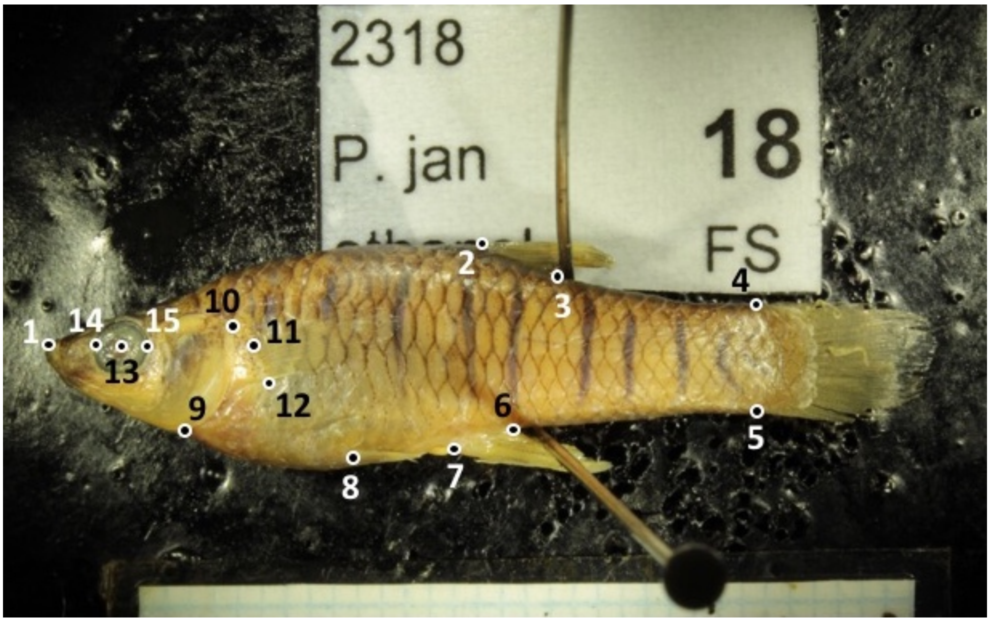

Figure A.1. Female *Phalloptychus januarius* with the 15 landmarks.

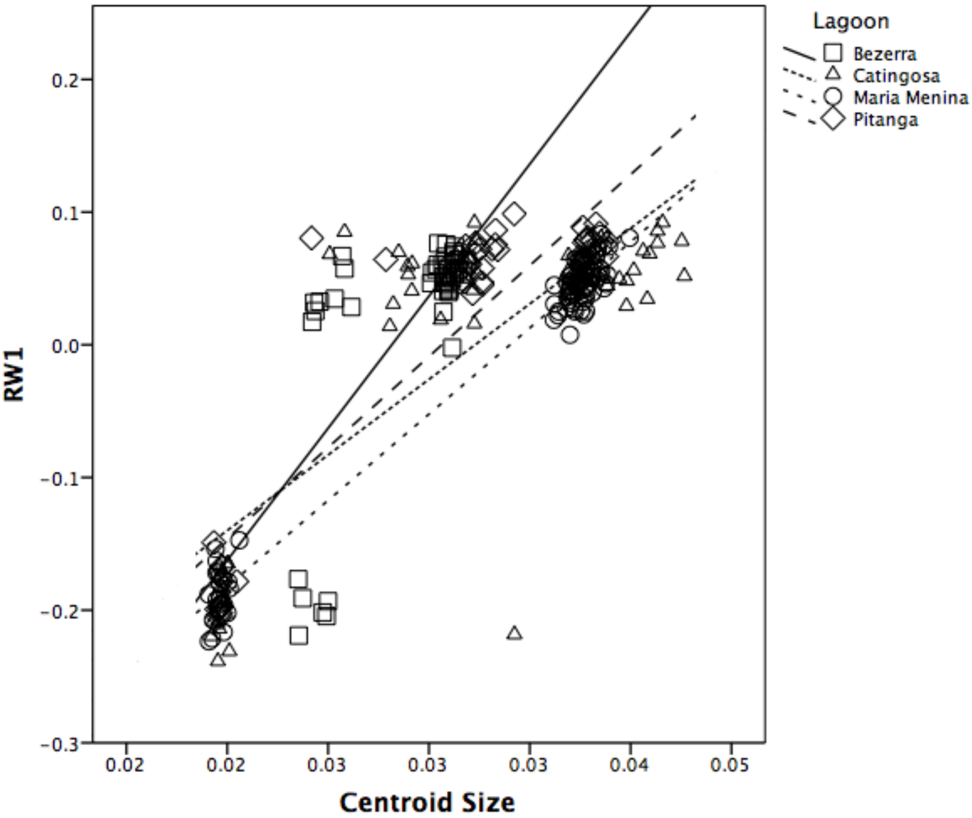

Figure A.2. Variation of RW1 as a function of centroid size in the 4 lagoons

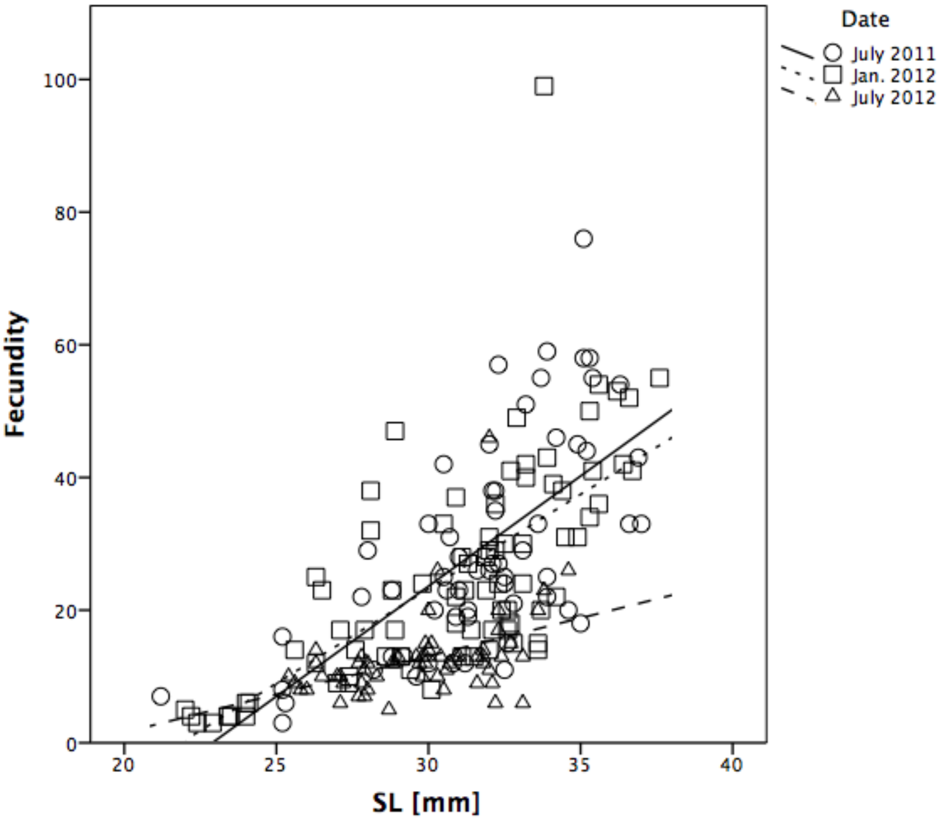

Figure A.3. Variation in fecundity as a function of SL between wet (July) and dry (January) seasons.

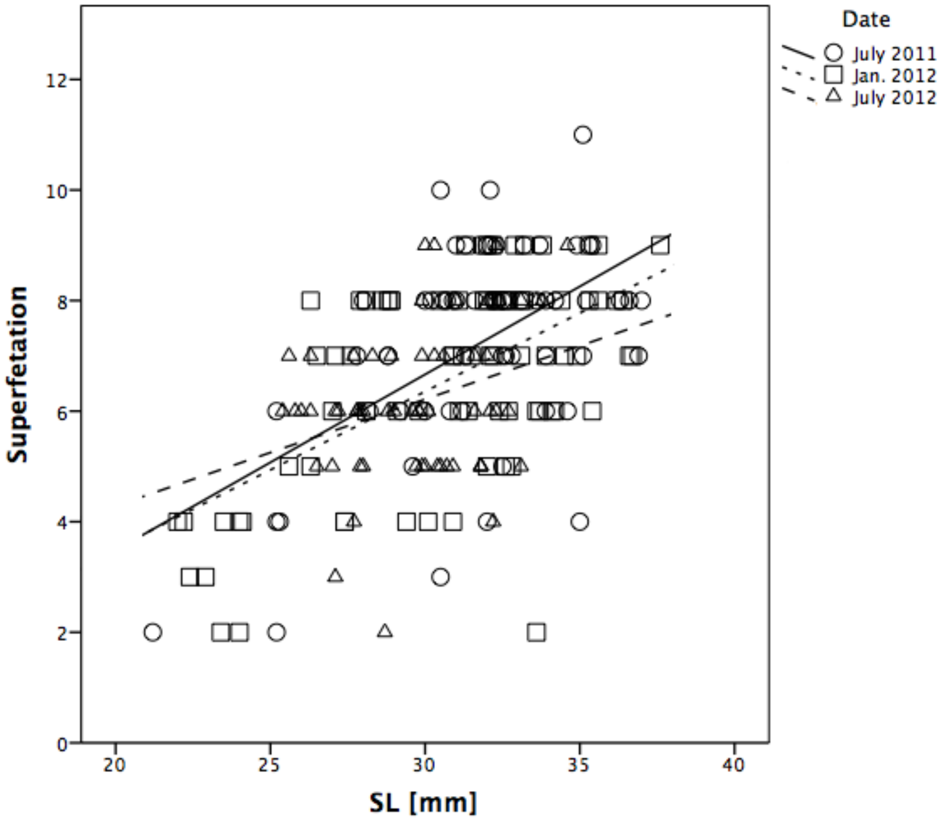

Figure A.4. Variation in superfetation as a function of SL between wet (July) and dry (January) seasons.

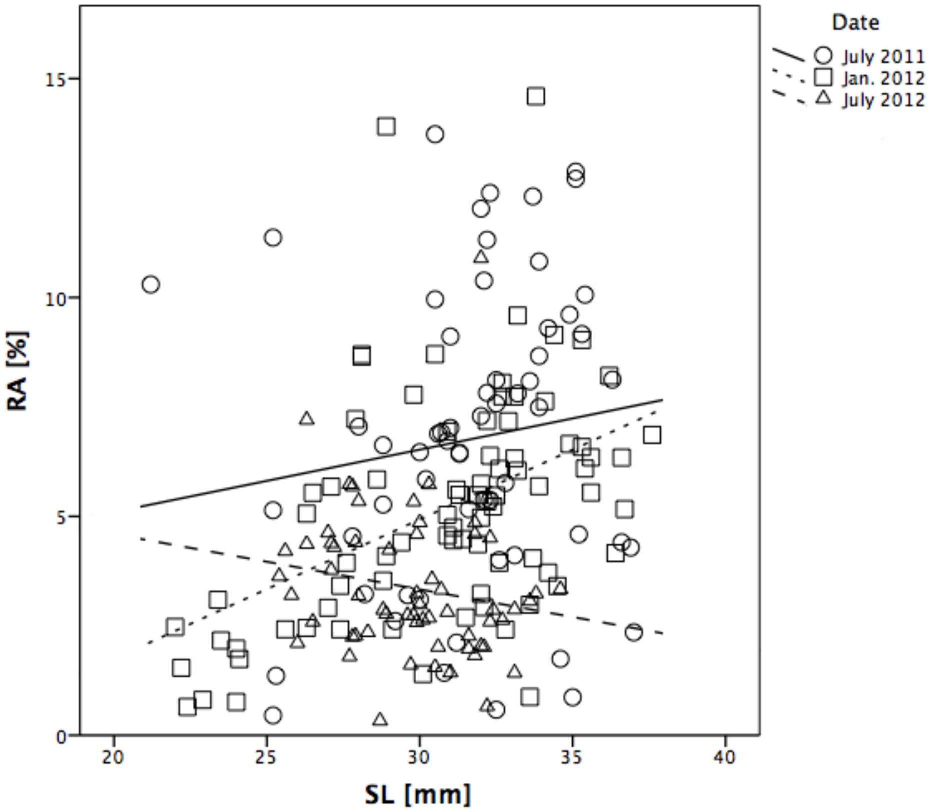

Figure A.5. Variation in RA as a function of SL between wet (July) and dry (January) seasons.

Appendix B: Supplementary material for Chapter 3 – A century later: adaptive plasticity and rapid evolution contribute to geographic variation in invasive mosquitofish

Appendix B1: Sampling sites and environmental parameters

Table B.1. Overview of climatic and environmental factors used to characterise sites at which invasive *G. holbrooki* were sampled. For the mean, maximum, and minimum temperatures, values represent averages over 120 days (± SD) preceding sampling.

Site number		Population name and code	Year	Latitude	Longitude	Distance from sea [m]	Dissolved oxygen [mg L^{-1}]	Conductivity [µS cm^{-1}]	Water temp. [°C]	Average mean temp. [°C]	Average maximum temp. [°C]	Average minimum temp. [°C]	Rainfall [mm] (days)
1	Italy	Lago di Garda - LdG	2013	45.4605	10.6113	136,892	1.54	560	17.0	10.33 ± 5.69	14.86 ± 6.56	6.39 ± 5.17	459 (58)
2	Italy	Comacchio – Com	2013	44.6908	12.1866	5,188	8.24	8,970	20.7	10.56 ± 5.33	14.48 ± 5.83	7.06 ± 5.11	477 (54)
3	Italy	Lago di Bolsena – LdB	2013	42.6314	11.9944	48,103	6.33	308	16.7	9.97 ± 4.58	13.73 ± 4.95	6.67 ± 4.56	329 (43)
4	Italy	Barletta – Blt	2013	41.3025	16.3526	403	4.01	6,850	19.5	13.62 ± 4.22	16.61 ± 4.47	11.17 ± 4.21	261 (40)
5	Italy	Torre Castiglione - TCa	2013	40.2890	17.8234	229	6.05	12,650	19.0	13.80 ± 4.23	16.89 ± 4.61	10.79 ± 4.07	217 (44)
6	Italy	Porto Cesareo – PCe	2013	40.2752	17.8770	356	11.41	16,520	23.0	13.92 ± 4.29	17.07 ± 4.66	10.86 ± 4.07	218 (44)
7	France	Briere – Bri	2013	47.3697	-2.3133	12,310	9.00	430	18.9	18.04 ± 3.45	22.97 ± 4.29	13.25 ± 3.30	157 (38)
8	France	La Ligneron – LLi	2013	46.7511	-1.9166	7,664	9.56	1,196	20.7	18.41 ± 3.56	23.04 ± 4.38	13.94 ± 3.29	143 (35)
9	France	La Charente – LCh	2013	45.9824	-0.9242	10,135	9.63	611	19.6	18.86 ± 3.83	23.40 ± 4.84	14.53 ± 3.38	195 (30)
10	France	Garonne – Gar	2013	45.0237	-0.5028	54,674	6.98	1,849	20.4	19.63 ± 4.27	25.12 ± 5.53	14.45 ± 3.57	282 (38)
11	France	Avignon – Avi	2013	43.7911	4.7547	42,719	3.28	458	20.0	21.48 ± 4.45	27.66 ± 5.25	15.38 ± 4.02	117 (17)
12	France	Arles – Arl	2013	43.6378	4.5549	20,623	10.10	560	21.6	22.12 ± 4.32	27.97 ± 4.96	16.37 ± 4.00	94 (16)
13	France	Montpellier – Mon	2013	43.5603	4.0298	1,731	8.10	47,100	25.0	21.79 ± 3.84	26.83 ± 4.24	16.80 ± 3.80	83 (14)
14	Spain	Zadorra – Zad	2013	42.8337	-2.7828	6,346	6.32	666	18.8	17.05 ± 4.70	24.13 ± 6.65	10.77 ± 3.96	180 (32)
15	Spain	Rio Ter – RTe	2013	42.0450	3.1744	1,874	6.51	890	23.5	20.58 ± 3.80	26.63 ± 4.34	14.67 ± 3.41	129 (23)
16	Spain	Barcelona – Bcn	2013	41.2628	1.6399	8,475	9.86	1,201	24.9	21.14 ± 3.80	26.70 ± 4.07	15.94 ± 3.75	114 (17)
17	Spain	Ebro Delta – EDe	2013	40.7072	0.5943	10,257	13.33	1,380	25.4	22.37 ± 3.54	27.63 ± 3.89	17.56 ± 3.46	116 (12)
18	Spain	Sagunt – Sag	2013	39.7286	-0.2074	2281	4.21	1,884	23.9	23.11 ± 3.47	27.76 ± 3.51	18.52 ± 3.59	58 (11)
19	Spain	Rio Júcar S – RJS	2013	39.1532	-0.2448	491	7.75	1,443	25.1	23.46 ± 3.21	27.64 ± 3.25	19.28 ± 3.39	54 (11)
20	Spain	Rio Segura – RSe	2013	38.1226	-0.6965	5,299	6.26	2,400	24.0	23.64 ± 3.35	28.56 ± 3.58	19.10 ± 3.49	35 (5)
21	Spain	Almanzora – Alm	2013	37.3138	-1.8924	13,846	11.06	2,820	28.2	23.07 ± 3.82	28.47 ± 4.29	18.06 ± 3.43	32 (6)

Table B.1 continued.

22	Spain	Guadalquivir – Gdq	2013	36.9378	-6.0975	31,258	0.00	16,300	32.0	24.67 ± 4.02	32.54 ± 5.42	16.94 ± 3.22	12 (4)
23	Spain	Guadiaro – Gdr	2013	36.3402	-5.3148	6,604	8.45	734	23.7	22.51 ± 3.21	26.79 ± 3.54	18.15 ± 3.10	18 (5)
24	Italy	Lago di Fimon W – LFW	2017	45.4708	11.5408	63,070	12.15	178	26.1	19.55 ± 5.17	25.29 ± 5.48	14.17 ± 4.74	391 (35)
25	Italy	Lago di Fimon S – LFS	2017	45.4635	11.5427	62,750	5.77	275	23.8	19.55 ± 5.17	25.29 ± 5.48	14.17 ± 4.74	391 (35)
26	Italy	Marina Grosseto – MGr	2017	42.7334	10.9652	136	7.26	4,020	24.5	21.30 ± 4.84	26.50 ± 5.00	16.23 ± 4.81	35 (8)
27	Italy	Grosseto – Gro	2017	42.7335	11.0413	5,310	3.72	1,583	23.7	21.39 ± 4.85	26.63 ± 5.04	16.28 ± 4.82	30 (6)
5	Italy	Torre Castiglione – TCa	2017	40.2888	17.8235	229	6.55	1,004	21.7	21.24 ± 5.06	24.79 ± 5.32	17.86 ± 4.86	73 (13)
6	Italy	Porto Cesareo – PCe	2017	40.2752	17.8769	356	13.70	1,287	30.7	21.66 ± 5.04	25.26 ± 5.27	18.20 ± 4.83	64 (11)
28	Spain	Gualta – Glt	2017	42.0311	3.1032	7,520	10.49	587	28.1	19.95 ± 4.63	25.98 ± 4.68	13.48 ± 5.08	79 (14)
29	Spain	Tortosa – Tor	2017	40.8078	0.5172	15,390	5.83	1,276	27.1	21.63 ± 4.73	28.13 ± 4.87	15.77 ± 5.03	73 (12)
30	Spain	L'Estanyol – Est	2017	39.7752	-0.1522	235	5.95	4,050	24.8	22.40 ± 4.57	27.36 ± 4.57	17.60 ± 4.75	45 (6)
18	Spain	Sagunt – Sag	2017	39.7308	-0.2080	2,281	18.00	1,369	28.1	22.40 ± 4.57	27.36 ± 4.57	17.60 ± 4.75	45 (6)
31	Spain	El Palmar – EPa	2017	39.3116	-0.3205	2,150	2.53	1,628	28.0	22.66 ± 4.43	27.26 ± 4.44	18.10 ± 4.62	54 (10)
32	Spain	Rio Júcar N – RJN	2017	39.1775	-0.2692	2,620	17.48	1,082	29.3	22.62 ± 4.38	27.16 ± 4.33	18.17 ± 4.56	36 (6)
33	Spain	Rio Vaca – RVa	2017	39.0606	-0.2183	1,440	4.71	1,514	30.7	22.07 ± 4.44	26.74 ± 4.40	17.61 ± 4.57	36 (7)
34	Spain	Lebrija – Leb	2017	36.9601	-6.0645	33,830	3.07	7,260	22.6	24.13 ± 4.01	31.99 ± 5.46	16.36 ± 3.26	93 (8)
22	Spain	Guadalquivir – Gdq	2017	36.9379	-6.0974	31,258	7.64	5,630	21.3	24.13 ± 4.01	31.99 ± 5.46	16.36 ± 3.26	93 (8)
35	Spain	Doñana N – DoN	2017	37.2260	-6.1412	43,650	4.65	2,970	25.5	24.67 ± 4.20	32.22 ± 5.57	17.12 ± 3.33	86 (7)
36	Spain	Doñana W – DoW	2017	37.2017	-6.2618	34,110	6.28	2,650	26.7	24.51 ± 4.15	31.93 ± 5.48	17.03 ± 3.30	86 (7)

Table B.2. Principal component analysis (PCA) with Varimax rotation on latitude, longitude, weather data and environmental parameters measured during sampling. For each principal component we present eigenvalues, % of variance explained and axis loadings for all variables included. Variables with axis loadings > 0.600 are highlighted in bold.

Principal component	EPC1	EPC2	EPC3
Eigenvalue	5.048	1.569	1.183
% var. explained	48.76	15.69	11.83
Cumulative % var. explained	48.76	62.45	74.28
Latitude	**-0.690**	0.034	0.265
Longitude	**-0.628**	0.479	-0.086
Dissolved oxygen	0.122	**0.660**	0.578
Conductivity	-0.002	0.305	**-0.823**
Water temperature	**0.741**	0.223	0.026
Distance from sea	-0.440	**-0.715**	0.105
Mean temperature	**0.957**	-0.155	0.055
Maximum temperature	**0.909**	-0.298	0.039
Minimum temperature	**0.936**	0.053	0.081
Rainfall	**-0.897**	-0.150	0.090

Appendix B2: Additional population genetic results

We used ARLEQUIN v 3.5 (Excoffier and Lischer 2010) to calculate pairwise F_{ST}-values between populations, as well as expected (H_E) and observed heterozygosities (H_O). FSTAT v 2.9.3 (Goudet 2001) was used to calculate allelic richness (*A*). We compared genetic richness (*A*) between North American and European samples using a two-samples *t*-test. To explore potential footprints of latitude on the population genetic structure (Riesch et al. 2018), we tested for a correlation between allelic richness (*A*) and latitude by means of a Pearson correlation.

Allelic richness (*A*) was significantly reduced in European populations compared to populations in their native range (t_{31} = 6.614, P < 0.001; Fig. B.1*a*). While populations in North America showed a strong decline in allelic richness (*A*) across latitude (Pearson's r = -0.66, P = 0.038; reanalysed from Riesch et al., 2018; Fig. B.1*b*), we could not find such a pattern in the genetically impoverished European populations (r = -0.58, P = 0.792; Fig. B1*c*). Descriptive statistics for site-specific means of standard indicators of genetic variability are provided in Table B.4.

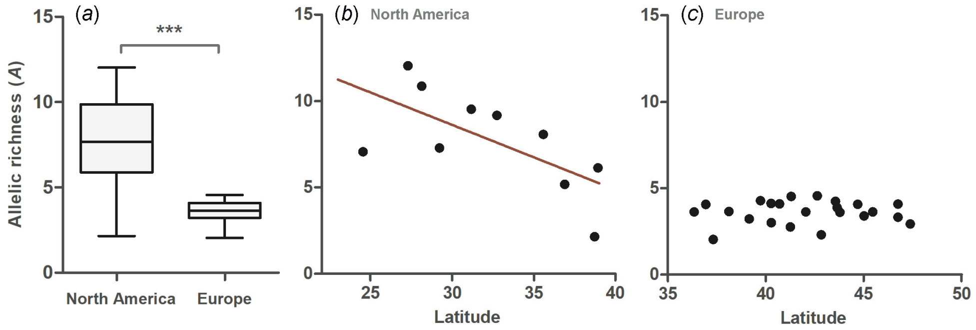

Figure B.1. (*a*) Allelic richness (*A*) differed between native and invasive mosquitofish populations (two-samples *t*-test; P < 0.001), (*b*) varied with latitude in native (North American) populations (Pearson's r = -0.66, P = 0.038), (*c*) but did not vary with latitude in invasive (European) ones (r = 0.06, P = 0.792). Figure created by J. Jourdan.

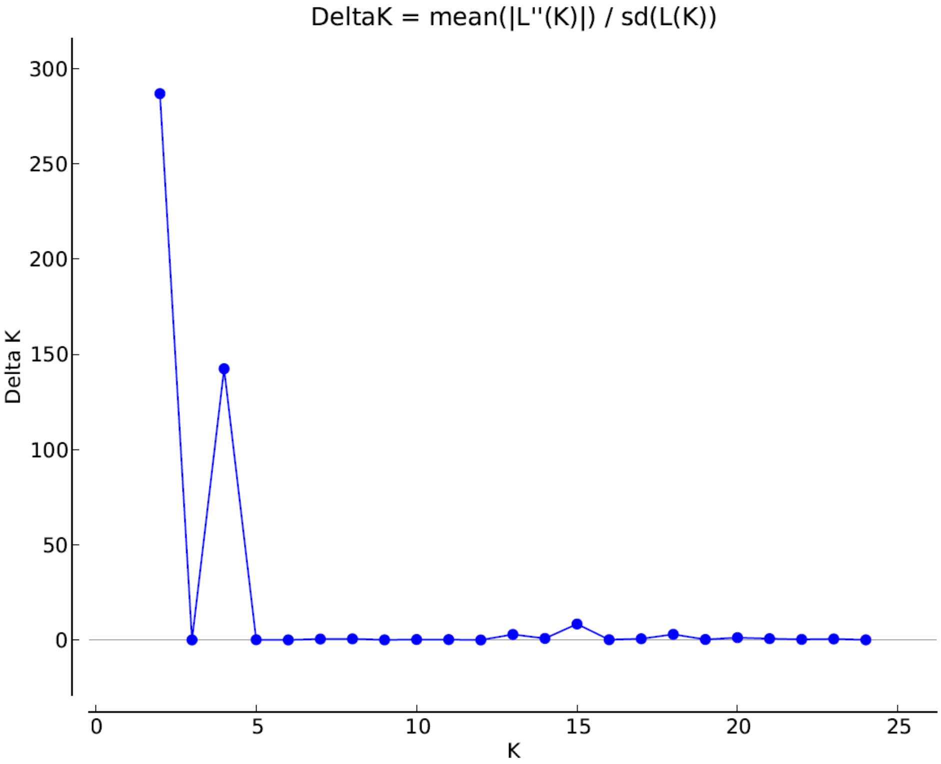

Figure B.2. Bayesian inference of the number of genetically distinct clusters (*K*) among the 33 genotyped populations using Δ*K* (Evanno et al. 2005). Figure created by J. Jourdan.

Table B.3. Combined Nei's and pair-wise F_{ST} distance matrix between native (North American; Riesch et al. 2018) and invasive (European) *G. holbrooki* populations. F_{ST}-values are reported in the upper half of the table (grey background), whereas Nei's distances are reported in the lower half of the table (white background).

Site number	NJ	DE	VA	NC	SC	GA	FLDB	FLME	FLPL	FLZT	**LdG**	**Com**	**LdB**	**Blt**	**TCa**	**PCe**	**Bri**	**LLi**	**LCh**	**Gar**	**Avi**	**Arl**	**Mon**	**Zad**	**RTe**	**Bcn**	**EDe**	**Sag**	**RJS**	**RSe**	**Alm**	**Gdq**	**Gdr**
NJ	-	0.329	0.172	0.122	0.216	0.21	0.242	0.168	0.191	0.325	0.296	0.218	0.227	0.210	0.294	0.257	0.306	0.300	0.231	0.316	0.234	0.287	0.257	0.354	0.242	0.281	0.217	0.209	0.287	0.258	0.441	0.258	0.257
DE	0.395	-	0.360	0.348	0.461	0.444	0.444	0.397	0.409	0.547	0.484	0.383	0.389	0.368	0.492	0.449	0.437	0.485	0.410	0.467	0.394	0.457	0.417	0.553	0.404	0.473	0.411	0.334	0.428	0.388	0.623	0.424	0.410
VA	0.412	0.429	-	0.125	0.252	0.250	0.263	0.224	0.230	0.355	0.317	0.213	0.219	0.194	0.306	0.269	0.279	0.313	0.225	0.261	0.199	0.278	0.251	0.377	0.233	0.279	0.233	0.189	0.239	0.202	0.443	0.239	0.235
NC	0.337	0.460	0.344	-	0.149	0.142	0.169	0.128	0.124	0.250	0.174	0.111	0.101	0.092	0.205	0.138	0.206	0.200	0.128	0.179	0.128	0.153	0.157	0.249	0.158	0.175	0.089	0.114	0.181	0.134	0.348	0.134	0.155
SC	0.526	0.701	0.565	0.438	-	0.065	0.126	0.120	0.069	0.202	0.244	0.218	0.206	0.199	0.281	0.235	0.278	0.255	0.224	0.274	0.229	0.243	0.236	0.309	0.257	0.290	0.208	0.254	0.319	0.290	0.415	0.233	0.267
GA	0.558	0.735	0.658	0.53	0.380	-	0.117	0.096	0.06	0.180	0.241	0.211	0.192	0.200	0.275	0.232	0.270	0.245	0.230	0.273	0.237	0.244	0.233	0.305	0.252	0.280	0.206	0.246	0.307	0.281	0.400	0.236	0.266
FLDB	0.554	0.701	0.615	0.50	0.456	0.447	-	0.08	0.056	0.162	0.263	0.227	0.216	0.209	0.279	0.242	0.277	0.266	0.230	0.264	0.242	0.238	0.203	0.332	0.259	0.297	0.214	0.250	0.306	0.276	0.400	0.231	0.261
FLME	0.450	0.655	0.563	0.439	0.425	0.404	0.372	-	0.035	0.149	0.244	0.206	0.198	0.190	0.260	0.222	0.272	0.261	0.204	0.252	0.224	0.228	0.195	0.332	0.248	0.288	0.180	0.222	0.266	0.246	0.385	0.226	0.245
FLPL	0.483	0.653	0.577	0.419	0.361	0.371	0.333	0.263	-	0.163	0.221	0.192	0.178	0.177	0.247	0.203	0.256	0.234	0.195	0.236	0.213	0.209	0.194	0.311	0.239	0.277	0.179	0.221	0.269	0.247	0.380	0.213	0.240
FLZT	0.678	0.831	0.725	0.620	0.516	0.493	0.502	0.462	0.511	-	0.349	0.321	0.309	0.319	0.397	0.353	0.389	0.371	0.338	0.369	0.349	0.360	0.303	0.416	0.369	0.393	0.318	0.356	0.410	0.383	0.503	0.341	0.372
LdG	0.523	0.510	0.501	0.345	0.547	0.608	0.576	0.543	0.544	0.699	-	0.088	0.083	0.074	0.227	0.094	0.250	0.143	0.133	0.191	0.149	0.120	0.145	0.254	0.210	0.258	0.159	0.174	0.245	0.241	0.393	0.230	0.214
Com	0.482	0.445	0.430	0.295	0.537	0.612	0.574	0.530	0.535	0.705	0.139	-	0.047	0.032	0.187	0.112	0.162	0.150	0.086	0.150	0.067	0.109	0.073	0.217	0.123	0.153	0.095	0.078	0.163	0.139	0.307	0.098	0.112
LdB	0.482	0.473	0.440	0.250	0.555	0.591	0.565	0.548	0.532	0.711	0.170	0.135	-	0.022	0.149	0.069	0.142	0.085	0.082	0.120	0.089	0.086	0.089	0.198	0.119	0.160	0.102	0.094	0.150	0.139	0.316	0.128	0.129
Blt	0.454	0.426	0.402	0.247	0.511	0.601	0.547	0.523	0.528	0.715	0.136	0.103	0.096	-	0.128	0.059	0.137	0.096	0.042	0.100	0.037	0.061	0.061	0.211	0.107	0.160	0.076	0.077	0.142	0.128	0.308	0.096	0.099
TCa	0.460	0.454	0.430	0.335	0.536	0.588	0.548	0.551	0.548	0.700	0.272	0.247	0.199	0.183	-	0.082	0.283	0.173	0.140	0.273	0.182	0.192	0.209	0.335	0.212	0.264	0.173	0.195	0.270	0.231	0.447	0.228	0.233
PCe	0.445	0.467	0.409	0.276	0.539	0.601	0.552	0.550	0.544	0.710	0.166	0.185	0.135	0.112	0.110	-	0.223	0.105	0.062	0.167	0.133	0.080	0.117	0.244	0.171	0.210	0.087	0.137	0.186	0.166	0.379	0.170	0.171
Bri	0.515	0.423	0.442	0.366	0.560	0.638	0.601	0.607	0.586	0.738	0.290	0.245	0.219	0.220	0.288	0.256	-	0.209	0.172	0.14	0.174	0.217	0.185	0.247	0.180	0.186	0.157	0.160	0.269	0.223	0.399	0.155	0.119
LLi	0.481	0.485	0.463	0.363	0.548	0.577	0.560	0.597	0.575	0.712	0.238	0.234	0.184	0.181	0.208	0.169	0.260	-	0.126	0.171	0.171	0.152	0.161	0.234	0.166	0.212	0.178	0.180	0.285	0.261	0.452	0.226	0.221
LCh	0.445	0.460	0.407	0.289	0.508	0.609	0.554	0.516	0.534	0.697	0.163	0.156	0.141	0.110	0.191	0.131	0.204	0.16	-	0.082	0.057	0.067	0.066	0.236	0.133	0.175	0.073	0.094	0.165	0.132	0.339	0.129	0.117
Gar	0.545	0.497	0.404	0.334	0.546	0.649	0.576	0.578	0.578	0.718	0.206	0.215	0.198	0.164	0.291	0.198	0.152	0.198	0.114	-	0.130	0.132	0.118	0.289	0.211	0.227	0.140	0.141	0.237	0.207	0.421	0.178	0.154
Avi	0.468	0.429	0.405	0.297	0.534	0.631	0.576	0.551	0.562	0.714	0.185	0.126	0.145	0.119	0.242	0.191	0.220	0.202	0.094	0.136	-	0.1	0.090	0.289	0.156	0.216	0.122	0.123	0.188	0.160	0.341	0.124	0.115
Arl	0.500	0.470	0.455	0.314	0.544	0.625	0.555	0.560	0.560	0.729	0.164	0.182	0.141	0.134	0.233	0.138	0.246	0.177	0.101	0.135	0.103	-	0.080	0.311	0.205	0.255	0.130	0.176	0.222	0.184	0.366	0.164	0.196
Mon	0.490	0.489	0.439	0.318	0.532	0.619	0.539	0.513	0.539	0.647	0.182	0.135	0.173	0.134	0.260	0.177	0.250	0.203	0.096	0.127	0.117	0.116	-	0.27	0.177	0.212	0.122	0.141	0.2	0.177	0.338	0.135	0.165
Zad	0.529	0.478	0.495	0.408	0.536	0.596	0.575	0.642	0.620	0.682	0.307	0.288	0.300	0.286	0.355	0.293	0.238	0.283	0.311	0.312	0.346	0.372	0.35	-	0.177	0.117	0.224	0.184	0.333	0.282	0.464	0.258	0.256
RTe	0.438	0.408	0.353	0.297	0.541	0.608	0.563	0.559	0.577	0.712	0.269	0.203	0.213	0.201	0.262	0.223	0.205	0.216	0.194	0.235	0.226	0.257	0.249	0.198	-	0.106	0.127	0.076	0.199	0.151	0.359	0.159	0.136
Bcn	0.475	0.462	0.393	0.309	0.558	0.618	0.582	0.613	0.606	0.716	0.335	0.233	0.258	0.242	0.289	0.260	0.200	0.272	0.243	0.239	0.279	0.305	0.276	0.159	0.131	-	0.167	0.112	0.275	0.198	0.441	0.177	0.179
EDe	0.478	0.484	0.437	0.269	0.535	0.634	0.564	0.545	0.555	0.710	0.247	0.183	0.200	0.183	0.243	0.165	0.201	0.275	0.167	0.205	0.211	0.249	0.214	0.274	0.180	0.209	-	0.071	0.161	0.131	0.337	0.076	0.096
Sag	0.427	0.380	0.364	0.258	0.542	0.612	0.559	0.541	0.550	0.713	0.236	0.161	0.189	0.167	0.235	0.196	0.213	0.274	0.181	0.223	0.213	0.268	0.236	0.203	0.122	0.150	0.102	-	0.102	0.070	0.307	0.116	0.084
RJS	0.511	0.464	0.422	0.344	0.634	0.656	0.599	0.548	0.571	0.734	0.314	0.251	0.226	0.244	0.335	0.263	0.307	0.342	0.281	0.288	0.286	0.304	0.292	0.347	0.261	0.321	0.240	0.182	-	0.055	0.328	0.195	0.151
RSe	0.477	0.404	0.368	0.286	0.586	0.638	0.578	0.572	0.567	0.710	0.321	0.227	0.216	0.236	0.293	0.248	0.268	0.344	0.247	0.269	0.244	0.268	0.285	0.284	0.201	0.227	0.206	0.123	0.111	-	0.299	0.145	0.120
Alm	0.575	0.505	0.543	0.467	0.644	0.670	0.597	0.624	0.638	0.766	0.379	0.331	0.343	0.362	0.419	0.392	0.362	0.462	0.364	0.405	0.365	0.386	0.395	0.345	0.330	0.379	0.350	0.272	0.281	0.244	-	0.359	0.287
Gdq	0.501	0.464	0.420	0.272	0.527	0.632	0.555	0.563	0.561	0.689	0.286	0.163	0.189	0.161	0.260	0.207	0.217	0.288	0.197	0.211	0.200	0.236	0.199	0.269	0.192	0.204	0.117	0.137	0.242	0.188	0.334	-	0.091
Gdr	0.491	0.451	0.421	0.304	0.571	0.656	0.585	0.590	0.584	0.741	0.287	0.183	0.197	0.193	0.279	0.219	0.171	0.290	0.209	0.204	0.210	0.272	0.259	0.242	0.190	0.196	0.138	0.135	0.204	0.164	0.260	0.116	-

Table B.4. Genetic diversity in European invasive eastern mosquitofish (*Gambusia holbrooki*). For each population (N = 20 individuals each) and locus, we report the observed (H_O) and expected heterozygosity (H_E) as well as allelic richness (A). Zero-values indicate monomorphic loci. See Table B.1 for population information.

Locus name	N° of alleles	Size range	Test	LdG	Com	LdB	Blt	TCa	PCe	Bri	LLi	LCh	Gar	Avi	Arl	Mon	Zad	RTe	Bcn	EDe	Sag	RJS	RSe	Alm	Gdq	Gdr	Mean across pop.
Gaaf10	27	136	H_O	0.35	0.55	0.45	0.60	0.05	0.30	0.50	0.45	0.55	0.70	0.20	0.40	0.35	0.50	0.45	0.40	0.70	0.30	0.65	0.75	0.35	0.50	0.95	0.48
			H_E	0.65	0.74	0.79	0.70	0.41	0.52	0.58	0.50	0.54	0.64	0.53	0.50	0.62	0.51	0.61	0.75	0.71	0.70	0.72	0.77	0.36	0.59	0.77	0.62
			A	3.95	5.00	5.00	4.00	2.00	4.95	4.95	3.00	4.00	3.95	3.95	3.00	3.95	2.00	4.00	4.00	4.95	4.00	5.00	5.00	2.00	5.95	5.00	4.07
Gaaf11	28	128	H_O	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.10	0.00	0.10	0.00	0.00	0.00	0.05	0.15	0.05	0.00	0.00	0.00	0.15	0.00	0.04
			H_E	0.00	0.39	0.33	0.34	0.00	0.26	0.47	0.00	0.44	0.51	0.42	0.56	0.61	0.00	0.00	0.31	0.75	0.24	0.00	0.33	0.00	0.64	0.10	0.29
			A	1.00	2.00	2.00	3.00	1.00	2.00	2.00	1.00	3.00	2.95	3.00	3.00	4.00	1.00	1.00	3.00	4.95	3.95	1.00	2.00	1.00	3.00	2.00	2.30
Gaaf13	35	196	H_O	0.75	0.95	0.85	0.95	0.80	0.85	0.55	0.65	1.00	0.65	0.80	0.70	0.80	0.60	0.80	0.65	0.70	0.75	0.70	0.80	0.45	0.75	0.90	0.76
			H_E	0.77	0.87	0.87	0.88	0.69	0.87	0.67	0.85	0.87	0.72	0.83	0.85	0.84	0.60	0.69	0.76	0.88	0.87	0.72	0.81	0.43	0.86	0.82	0.78
			A	6.70	10.90	8.90	10.90	5.85	10.00	4.95	7.00	8.90	5.85	6.95	8.00	7.90	4.90	5.95	5.90	9.85	11.80	5.90	9.80	3.90	9.85	7.95	7.77
Gafµ3	39	138	H_O	0.70	0.75	0.55	0.65	0.40	0.65	0.45	0.55	0.70	0.60	0.90	0.55	0.70	0.30	0.70	0.20	0.55	0.75	0.70	0.80	0.10	0.55	0.75	0.59
			H_E	0.85	0.70	0.81	0.83	0.57	0.78	0.73	0.77	0.83	0.74	0.83	0.86	0.82	0.45	0.67	0.26	0.60	0.65	0.70	0.77	0.10	0.61	0.74	0.68
			A	6.95	6.90	7.95	6.00	4.90	5.00	4.95	5.00	7.90	6.90	7.90	7.95	7.00	2.95	3.95	2.00	4.90	4.00	3.95	5.00	2.00	4.00	5.95	5.39
Gaaf16	23	136	H_O	0.00	0.00	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01
			H_E	0.33	0.26	0.30	0.19	0.26	0.19	0.10	0.26	0.19	0.00	0.10	0.10	0.19	0.00	0.14	0.00	0.00	0.00	0.10	0.00	0.00	0.00	0.00	0.12
			A	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	1.00	2.00	2.00	2.00	1.00	2.00	1.00	1.00	1.00	2.00	1.00	1.00	1.00	1.00	1.61
Gaaf7	25	120	H_O	0.55	0.50	0.60	0.55	0.50	0.55	0.35	0.35	0.40	0.50	0.45	0.30	0.35	0.05	0.55	0.25	0.45	0.60	0.55	0.75	0.00	0.30	0.45	0.45
			H_E	0.52	0.51	0.55	0.55	0.59	0.69	0.41	0.30	0.43	0.39	0.48	0.33	0.41	0.05	0.71	0.30	0.61	0.66	0.56	0.59	0.00	0.58	0.55	0.47
			A	2.95	2.00	3.00	3.00	3.00	4.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	1.95	4.95	2.00	3.95	4.00	3.00	3.00	1.00	3.00	3.00	2.69
Gaaf22	46	129	H_O	0.60	0.75	0.55	0.75	0.30	0.65	0.50	0.60	0.75	0.55	0.55	0.65	0.65	0.35	0.85	0.40	0.65	0.95	0.60	0.40	0.30	0.90	0.45	0.60
			H_E	0.83	0.82	0.80	0.85	0.49	0.76	0.59	0.72	0.71	0.65	0.74	0.76	0.64	0.36	0.76	0.55	0.71	0.81	0.59	0.43	0.39	0.72	0.45	0.66
			A	6.00	6.00	6.95	7.85	4.95	6.95	4.00	4.95	6.00	4.95	5.90	5.95	5.95	4.00	5.00	3.00	4.00	6.95	3.00	2.00	2.00	5.95	2.00	4.97
Gafµ2	30	78	H_O	0.00	0.6.	0.50	0.45	0.35	0.55	0.55	0.35	0.35	0.55	0.50	0.45	0.45	0.70	0.40	0.55	0.60	0.35	0.15	0.05	0.20	0.30	0.30	0.42
			H_E	0.00	0.56	0.56	0.58	0.36	0.48	0.65	0.45	0.48	0.51	0.53	0.41	0.41	0.68	0.47	0.48	0.62	0.34	0.35	0.05	0.55	0.45	0.42	0.45
			A	1.00	3.00	3.95	3.00	2.00	2.00	3.00	2.00	2.95	2.00	3.00	2.00	2.00	3.00	3.95	2.00	3.00	3.00	3.95	1.95	3.00	3.95	4.00	2.77
Gaaf9	17	92	H_O	0.30	0.25	0.25	0.25	0.00	0.00	0.00	0.00	0.25	0.05	0.55	0.05	0.25	0.00	0.20	0.00	0.35	0.05	0.00	0.05	0.00	0.40	0.30	0.22
			H_E	0.47	0.45	0.30	0.41	0.00	0.00	0.10	0.00	0.27	0.05	0.51	0.05	0.23	0.00	0.27	0.00	0.34	0.05	0.00	0.05	0.00	0.43	0.43	0.19
			A	2.00	2.00	2.00	2.00	1.00	1.00	2.00	1.00	2.95	1.95	2.00	1.95	2.95	1.00	3.00	1.00	2.95	1.95	1.00	1.95	1.00	2.00	2.00	1.85
Gafµ6	20	120	H_O	0.35	0.15	0.05	0.20	0.05	0.20	0.00	0.20	0.10	0.05	0.10	0.00	0.20	0.00	0.15	0.00	0.25	0.10	0.50	0.30	0.00	0.10	0.25	0.14
			H_E	0.38	0.55	0.56	0.59	0.55	0.34	0.19	0.66	0.69	0.36	0.47	0.49	0.41	0.49	0.54	0.51	0.63	0.57	0.54	0.51	0.33	0.43	0.47	0.49
			A	3.00	3.00	3.00	3.00	3.00	3.00	2.00	4.00	4.00	2.00	2.00	2.00	3.00	2.00	3.00	2.00	3.00	3.95	2.95	2.00	2.00	2.00	3.00	2.73
Gaaf15	13	72	H_O	0.35	0.35	0.50	0.45	0.35	0.45	0.30	0.30	0.50	0.30	0.75	0.55	0.45	0.20	0.45	0.30	0.55	0.70	0.35	0.55	0.60	0.20	0.60	0.44
			H_E	0.41	0.50	0.55	0.51	0.36	0.45	0.43	0.27	0.49	0.51	0.52	0.50	0.50	0.19	0.38	0.34	0.47	0.57	0.30	0.51	0.51	0.33	0.49	0.44
			A	2.00	2.00	3.00	2.00	2.00	2.00	2.00	3.00	2.00	2.00	2.95	2.95	2.00	2.00	2.95	2.95	2.95	3.00	2.00	3.00	2.00	2.00	2.00	2.38
Gafµ7	30	90	H_O	0.50	0.70	0.75	0.75	0.50	0.75	0.05	0.50	0.50	0.15	0.75	0.45	0.85	0.55	0.80	0.80	0.65	0.70	0.50	0.75	0.00	0.40	0.55	0.56
			H_E	0.63	0.73	0.81	0.86	0.57	0.85	0.52	0.56	0.71	0.41	0.71	0.68	0.73	0.70	0.73	0.67	0.78	0.78	0.62	0.85	0.10	0.70	0.75	0.67
			A	4.90	6.90	7.00	8.00	4.90	7.95	3.00	3.95	5.85	2.00	4.90	4.00	5.00	3.95	5.90	4.95	5.50	5.95	4.90	7.95	2.00	4.95	6.90	5.29
Mf13	12	32	H_O	0.35	0.35	0.45	0.40	0.25	0.35	0.00	0.30	0.40	0.45	0.30	0.55	0.40	0.00	0.00	0.00	0.45	0.65	0.40	0.35	0.15	0.25	0.35	0.38
			H_E	0.67	0.41	0.51	0.39	0.38	0.54	0.00	0.50	0.53	0.56	0.30	0.65	0.47	0.00	0.00	0.00	0.48	0.48	0.50	0.48	0.14	0.30	0.36	0.38
			A	4.00	2.00	3.00	2.00	3.00	3.00	1.00	3.00	3.00	4.90	2.95	3.95	2.95	1.00	1.00	1.00	2.00	2.00	3.00	2.00	2.00	2.00	2.00	2.47
Gafµ1	9	70	H_O	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
			H_E	0.10	0.00	0.00	0.00	0.10	0.00	0.00	0.00	0.00	0.19	0.00	0.00	0.49	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04
			A	2.00	1.00	1.00	1.00	2.00	1.00	1.00	1.00	1.00	2.00	1.00	1.00	2.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.17
Gafµ4	61	170	H_O	0.80	0.55	0.65	0.75	0.75	0.50	0.60	0.25	0.65	0.75	0.90	0.80	0.75	0.50	0.40	0.65	0.30	0.85	0.60	0.75	0.50	0.70	0.50	0.63
			H_E	0.83	0.83	0.91	0.90	0.80	0.80	0.85	0.75	0.68	0.80	0.80	0.87	0.87	0.73	0.79	0.80	0.88	0.88	0.78	0.82	0.61	0.90	0.81	0.81
			A	7.90	10.80	11.90	12.75	9.75	8.90	6.00	7.95	8.85	9.80	5.95	10.80	14.50	4.00	8.95	6.90	9.90	10.85	7.90	9.80	5.85	13.65	8.80	9.24
Mean across loci			H_O	0.43	0.46	0.44	0.48	0.33	0.45	0.30	0.38	0.44	0.39	0.48	0.40	0.41	0.38	0.48	0.39	0.49	0.52	0.48	0.49	0.27	0.42	0.49	
			H_E	0.50	0.55	0.58	0.57	0.41	0.50	0.42	0.44	0.52	0.47	0.52	0.51	0.55	0.32	0.45	0.38	0.56	0.51	0.434	0.46	0.23	0.50	0.48	
			A	3.77	4.36	4.71	4.70	3.42	4.25	2.99	3.39	4.29	3.62	3.76	4.04	4.48	2.38	3.77	2.85	4.29	4.49	3.37	3.83	2.12	4.29	3.77	

Appendix B3: Extended results – phenotypic variation in wild-caught mosquitofish

Phenotypic variation between populations and genetic clusters

In the analysis of male and female body size we found significant effects of the factors ‘sex’ (MANCOVA; $F_{3,\,1252} = 8{,}842.64$, $P < 0.001$, $\eta_p^2 = 0.955$), ‘year’ ($F_{3,\,1252} = 17.11$, $P < 0.001$, $\eta_p^2 = 0.039$), ‘population(cluster)’ ($F_{102,\,3750} = 7.83$, $P < 0.001$, $\eta_p^2 = 0.175$) and ‘cluster’ ($F_{3,\,1252} = 11.35$, $P < 0.001$, $\eta_p^2 = 0.026$), as well as the covariate ‘centroid size’ ($F_{3,\,1252} = 8.63$, $P < 0.001$, $\eta_p^2 = 0.020$) and the interactions ‘sex × population(cluster)’ ($F_{102,\,3750} = 4.75$, $P < 0.001$, $\eta_p^2 = 0.114$), ‘year × cluster’ ($F_{3,\,1252} = 5.63$, $P = 0.001$, $\eta_p^2 = 0.013$) and ‘year × population(cluster)’ ($F_{6,\,2504} = 5.41$, $P < 0.001$, $\eta_p^2 = 0.013$; Table B.8*a*). Sexual dimorphism was the greatest source of body shape diversity (Fig. B.3), but the extent of sexual dimorphism changed between populations (‘sex × population(cluster)’-effect). Furthermore, body size varied between populations and between Spanish and French/Italian fish (‘population(cluster)’- and ‘cluster’-effects respectively), however these differences varied depending on the year in which the mosquitofish were sampled.

Similarly, male life-history traits were significantly affected by ‘SL’ ($F_{3,\,544} = 510.22$, $P < 0.001$, $\eta_p^2 = 0.738$), but also varied between populations (‘population’-effect; $F_{102,\,1639} = 4.52$, $P < 0.001$, $\eta_p^2 = 0.220$), genetic clusters (‘cluster’-effect; $F_{3,\,544} = 3.15$, $P = 0.025$, $\eta_p^2 = 0.017$), and sampling years (‘year’-effect; $F_{3,\,544} = 12.93$, $P < 0.001$, $\eta_p^2 = 0.067$). Moreover, male life histories scaled differently with body size in different populations (‘SL × population(cluster)’-effect; $F_{105,\,1630} = 1.35$, $P = 0.013$, $\eta_p^2 = 0.080$), and the differences between clusters varied between sampling years (‘year × cluster’-effect; $F_{3,\,544} = 2.69$, $P = 0.046$, $\eta_p^2 = 0.015$; Table B.8*b*).

Lastly, in the analysis on female life-history traits we found significant effects of both the covariates ‘SL’ ($F_{6,\,633} = 655.89$, $P < 0.001$, $\eta_p^2 = 0.861$) and ‘embryonic stage of development ($F_{6,\,633} = 7.08$, $P < 0.001$, $\eta_p^2 = 0.063$). Female life histories also varied depending on ‘population(cluster)’ ($F_{198,\,3753} = 7.70$, $P < 0.001$, $\eta_p^2 = 0.265$), the genetic cluster ($F_{6,\,633} = 14.97$, $P < 0.001$,

η_p^2 = 0.124) and sampling years ($F_{6,\ 633}$ = 8.45, P < 0.001, η_p^2 = 0.074). Finally, life-history traits scaled differently with body size between populations ($F_{198,\ 3753}$ = 3.11, P < 0.001, η_p^2 = 0.139) and genetic clusters ($F_{6,\ 633}$ = 5.04, P < 0.001, η_p^2 = 0.046; Table B.8*c*).

Body-size variation

In the ANCOVA on body size (SL), the factors 'sex' ($F_{1,\ 1321}$ = 2,752.60, P < 0.001, η_p^2 = 0.676) and 'year' ($F_{1,\ 1321}$ = 27.55, P < 0.001, η_p^2 = 0.020) had significant effects, as did the covariates 'EPC1' ($F_{1,\ 1321}$ = 5.38, P = 0.022, η_p^2 = 0.004), 'EPC2' ($F_{1,\ 1321}$ = 5.26, P = 0.022, η_p^2 = 0.004) and the interactions 'sex × EPC1' ($F_{1,\ 1321}$ = 23.43, P < 0.001, η_p^2 = 0.017), 'sex × EPC3' ($F_{1,\ 1321}$ = 3.95, P = 0.047, η_p^2 = 0.003), 'year × EPC1' ($F_{1,\ 1321}$ = 8.11, P = 0.004, η_p^2 = 0.006) and 'year × 'EPC2' ($F_{1,\ 1321}$ = 12.99, P < 0.001, η_p^2 = 0.010), while 'EPC3' did not have a significant effect ($F_{1,\ 1321}$ = 0.93, P = 0.334, η_p^2 = 0.001). Based on our measure of effect strength (η_p^2), 'sex' had the strongest overall effect, with females being larger than males. Fish sampled in 2013 were also larger than those sampled in 2017 ('year'-effect). Among the effects of environmental variables (EPCs), fish had larger bodies in northern than in southern populations ('EPC1'-effect), and this effect was more pronounced in males than in females ('sex × EPC1'-effect; Fig. 3.3*b*). The 'EPC2'-effect suggested that in high-oxygen, coastal environments fish tended to be smaller than in low-oxygen, inland conditions, but this response was much more pronounced in 2017 than in 2013 ('year × EPC2'-effect).

Body-shape variation

In the analysis on body-shape variation, all factors and the covariates 'EPC1' and 'EPC3' had significant effects (Table 3.1*a*). Nonetheless, 'sex' had the strongest overall effect (η_p^2 = 0.962), while the importance of the other significant effects was considerably lower (all η_p^2 < 0.050). The main source of body-shape variation regarding sexual dimorphism concerned the enlarged abdominal region in females, while in males the anal fin (modified into the gonopodium) was shifted towards the anterior. Our analysis also highlighted the presence of allometric effects due to body size differences (‘centroid size’-effect). Additionally, body shape changed in response to environmental variation and between sampling years. However, the responses to climate variation differed between males and females (‘sex × EPC1’-effect), and between 2013 and 2017 (‘year × EPC1’-effect), as did the responses to differences in conductivity (‘year × EPC3’-effect).

Post-hoc ANCOVAs (Table 3.2; $\alpha' = 0.017$, corrected for multiple testing) revealed that ‘sex’, ‘centroid size’ and ‘year × EPC1’ significantly affected RW1-3. ‘Year’, ‘EPC1’ and ‘year × EPC3’ had significant effects on RW1 and RW3; ‘EPC3’ on RW1 and RW2; and ‘sex × EPC1’ significantly affected RW3. The ‘sex’-effect reflected that, besides the presence of a gonopodium, males were also characterised by more streamlined bodies and relatively larger heads than females. Bigger fish had deeper and rounder bodies than smaller ones (‘centroid size’-effects), as had fish from 2013 when compared to fish from 2017 (‘year’-effect). When considering environmental variation, in colder climates (i.e., northern and Italian populations) males had rounder and deeper bodies compared to fish from warmer climates (i.e., southern and Spanish populations), whereas females had deeper and rounder bodies in southern, rather than northern populations (‘sex × EPC1’-effect). Moreover, the ‘year × EPC1’-effect suggests that these responses were more pronounced in 2013 than in 2017. Lastly, in low-conductivity environments fish had deeper bodies than in high-conductivity ones (‘EPC3’-effect), but this effect was much stronger in fish sampled in 2017 than in those sampled in 2013 (‘year × EPC3’-effect).

Life-history variation

In the analysis on male life-history traits we found significant effects of SL, ‘year’, ‘EPC1’, ‘EPC3’ and ‘Year × EPC1’, while EPC2 had no significant effect (Table 3.1*b*). ‘SL’ had the strongest effect ($\eta_p^2 = 0.838$), followed by ‘year’ ($\eta_p^2 = 0.258$), but ‘EPC1’, ‘EPC3’ and ‘Year × EPC1’ all had relatively weak effects ($\eta_p^2 \leq 0.100$ in all cases). *Post-hoc* ANCOVAs (Table 3.3; $\alpha' = 0.017$) uncovered a significant effect of body size (SL) on lean weight, fat content and GSI, with bigger males having higher lean weights and fat contents but lower GSI. ‘Year’ had a significant effect on fat content and GSI, as males were characterised by higher fat content and reproductive investment in 2017 than in 2013. Moreover, ‘EPC1’ had a significant effect on lean weight and GSI, as males from northern and colder populations were heavier and had higher GSI than those from southern/warmer populations. ‘EPC3’ had a significant effect on lean weight and fat content, showing that in high-conductivity populations males were heavier but had lower fat content than in low-conductivity ones. Lastly, ‘SL x EPC3’ significantly affected GSI, with bigger males having a higher GSI compared to smaller males in high-, but not in low-conductivity populations.

In the analysis of female life histories, the factor ‘year’, all covariates, as well as the interactions ‘year × EPC1-3’ and ‘SL × EPC1-3’ had significant effects. Based on η_p^2, ‘SL’ had by far the strongest effect ($\eta_p^2 = 0.937$), followed by ‘embryonic stage of development’, ‘EPC3’, ‘year × EPC3’ and ‘year’ and ($\eta_p^2 = 0.144$, 0.138, 0.131 and 0.126, respectively). ‘EPC1’, ‘EPC2’, ‘year × EPC1’, ‘year × EPC2’, and ‘SL × EPCs’, while significant, were of lower importance ($\eta_p^2 < 0.100$; Table 3.1*c*). This suggests that female life-history traits changed during offspring development (‘embryonic stage of development’-effect) and as a function of body size (SL-effect), differed between years, and in response to different environmental conditions. However, these responses to climate varied between 2013 and 2017 (‘year × EPC’-effects), and bigger females responded to environmental variation differently than smaller ones (‘SL × EPC’-effects). *Post-hoc* ANCOVAs (Table 3.4; $\alpha' = 0.008$) revealed that female lean weight, fecundity, embryo lean weight, and RA all increased as females became bigger (SL-effects). ‘Embryonic stage of development’ affected embryo fat and lean weight, as embryos decreased in weight and lost body fat during development, and fecundity, which decreased as embryos progressed in development. ‘Year’ significantly affected lean weight, adult fat content and embryo fat content and RA, as fish were lighter and had reduced reproductive investment in 2017 than in 2013 but had increased adult and embryo fat contents. Considering the effects of environmental variation, ‘EPC1’ significantly affected RA, ‘EPC2’ offspring lean weight, and ‘EPC3’ affected adult fat content and RA, as well as embryo fat content. Females from colder populations invested more into reproduction (RA) than those from warmer populations (‘EPC1’-effect). The ‘EPC2’ effect suggested that females in high-oxygen environments tended to have bigger offspring than in low-oxygen environments, while female in low-conductivity environments had increased body and embryo fat and RA (‘EPC3’-effect).

Regarding the interaction effects, adult fat content was significantly affected by ‘SL × EPC1’, while ‘SL × EPC3’ affected adult and embryo weight, fecundity and RA. Larger females had higher fat content in warmer (southern) populations, while the difference was greatly reduced in smaller females (‘SL × EPC1’- effect). Moreover, only in high-conductivity populations did larger females have relatively higher body weight, fecundity, and offspring size compared to small-bodied females. Lastly, ‘year × EPC1’ affected RA, ‘year × EPC2’ affected fecundity, embryo weight and fat content, and ‘year × EPC3’ affected fecundity, RA, and embryo fat content. In 2017, females in colder populations were characterised by increased RA when compared with warmer populations, but this effect disappeared in 2013 (‘year × EPC1’-effect). Similarly, the ‘year × EPC2’-effect suggests that in high-oxygen environments females were characterised by reduced fecundity and increased embryo weight and fat content in 2013, while this pattern was reversed in 2017. The ‘year × EPC3’-effect suggests that in high-conductivity environments females had higher fecundity and RA, but lower embryo fat content in 2017, but these relationships were reversed in 2013.

Table B.5. Overview of relative warps (RWs) used for the body-shape analysis of wild-caught mosquitofish. For each RW, eigenvalues and the % of variance explained are presented.

RW	Eigenvalue	% variance explained	Cumulative % var. explained
RW1	2.6306	87.30	87.30
RW2	0.4061	2.08	89.30
RW3	0.3813	1.83	91.21

Table B.6. Descriptive statistics (mean ± s.e.m.) of life-history traits of wild-caught male *G. holbrooki*.

Site number		Population name and code	Year	*N*	SL [mm]	Lean weight [g]	Fat content [%]	GSI [%]
1	Italy	Lago di Garda – LdG	2013	5	21.2 ± 0.6	0.038 ± 0.004	0.28 ± 0.14	2.57 ± 0.19
2	Italy	Comacchio – Com	2013	22	23.3 ± 0.3	0.051 ± 0.003	0.75 ± 0.06	3.16 ± 0.10
3	Italy	Lago di Bolsena – LdB	2013	15	22.0 ± 0.6	0.038 ± 0.003	1.52 ± 0.17	2.21 ± 0.07
4	Italy	Barletta – Blt	2013	20	21.3 ± 0.3	0.033 ± 0.002	1.29 ± 0.13	1.69 ± 0.07
5	Italy	Torre Castiglione – TCa	2013	24	21.5 ± 0.2	0.037 ± 0.001	0.70 ± 0.08	1.91 ± 0.08
6	Italy	Porto Cesareo - PCe	2013	16	20.3 ± 0.3	0.031 ± 0.001	0.30 ± 0.09	2.32 ± 0.12
7	France	Briere – Bri	2013	12	19.4 ± 0.4	0.027 ± 0.002	0.16 ± 0.09	2.08 ± 0.15
8	France	La Ligneron – LLi	2013	22	18.9 ± 0.4	0.026 ± 0.002	0.51 ± 0.17	2.25 ± 0.07
9	France	La Charente – LCh	2013	16	21.1 ± 0.4	0.030 ± 0.002	4.17 ± 0.41	2.06 ± 0.12
10	France	Garonne – Gar	2013	21	19.4 ± 0.4	0.025 ± 0.002	3.51 ± 0.45	2.58 ± 0.08
11	France	Avignon – Avi	2013	13	18.8 ± 0.5	0.027 ± 0.003	0.67 ± 0.15	2.78 ± 0.17
12	France	Arles – Arl	2013	24	19.4 ± 0.5	0.026 ± 0.002	1.73 ± 0.26	1.73 ± 0.09
13	France	Montpellier – Mon	2013	19	18.6 ± 0.4	0.022 ± 0.002	0.28 ± 0.10	2.60 ± 0.11
14	Spain	Zadorra – Zad	2013	25	18.6 ± 0.3	0.025 ± 0.002	0.32 ± 0.09	2.14 ± 0.08
15	Spain	Rio Ter – Rte	2013	12	20.3 ± 0.6	0.027 ± 0.003	1.37 ± 0.33	1.64 ± 0.13
16	Spain	Barcelona – Bcn	2013	19	20.2 ± 0.3	0.030 ± 0.002	0.66 ± 0.12	2.17 ± 0.12
17	Spain	Ebro Delta – EDe	2013	17	19.1 ± 0.5	0.024 ± 0.002	1.82 ± 0.37	2.27 ± 0.08
18	Spain	Sagunt – Sag	2013	19	19.2 ± 0.6	0.023 ± 0.003	0.77 ± 0.27	2.00 ± 0.11
19	Spain	Rio Júcar S – RJS	2013	9	19.9 ± 0.5	0.022 ± 0.003	0.95 ± 0.19	2.07 ± 0.10

Table B.6 continued.

20	Spain	Rio Segura – RSe	2013	23	19.0 ± 0.3	0.023 ± 0.001	0.73 ± 0.11	2.28 ± 0.11
21	Spain	Almanzora – Alm	2013	9	18.3 ± 0.2	0.020 ± 0.001	0.59 ± 0.16	1.84 ± 0.09
22	Spain	Guadalquivir – Gdq	2013	23	18.4 ± 0.2	0.021 ± 0.001	0.74 ± 0.17	1.83 ± 0.08
23	Spain	Guadiaro – Gdr	2013	19	18.9 ± 0.6	0.026 ± 0.003	0.56 ± 0.12	2.04 ± 0.08
24	Italy	Lago di Fimon W – LFW	2017	18	19.0 ± 0.4	0.023 ± 0.002	4.33 ± 0.92	2.73 ± 0.13
25	Italy	Lago di Fimon S – LFS	2017	11	18.1 ± 0.4	0.022 ± 0.002	4.10 ± 1.16	3.29 ± 0.11
26	Italy	Marina Grosseto – MGr	2017	22	18.0 ± 0.2	0.021 ± 0.001	3.05 ± 0.68	3.56 ± 0.15
27	Italy	Grosseto – Gro	2017	6	17.5 ± 0.6	0.019 ± 0.002	1.56 ± 1.03	4.51 ± 0.40
5	Italy	Torre Castiglione – TCa	2017	21	20.9 ± 0.3	0.033 ± 0.002	1.96 ± 0.52	2.52 ± 0.16
6	Italy	Porto Cesareo – PCe	2017	5	15.7 ± 0.6	0.012 ± 0.001	1.25 ± 1.25	2.99 ± 0.35
28	Spain	Gualta – Glt	2017	22	17.9 ± 0.2	0.019 ± 0.001	2.38 ± 0.79	2.94 ± 0.14
29	Spain	Tortosa – Tor	2017	10	19.4 ± 0.4	0.027 ± 0.002	2.82 ± 0.82	3.25 ± 0.23
30	Spain	L’Estanyol – Est	2017	8	17.5 ± 0.2	0.018 ± 0.001	5.48 ± 1.82	3.05 ± 0.50
18	Spain	Sagunt – Sag	2017	3	18.2 ± 0.5	0.019 ± 0.002	3.33 ± 3.33	2.87 ± 0.22
31	Spain	El Palmar - EPa	2017	18	19.2 ± 0.7	0.028 ± 0.003	3.70 ± 0.95	2.98 ± 0.21
32	Spain	Rio Júcar N – RJN	2017	11	17.4 ± 0.4	0.020 ± 0.002	3.58 ± 1.16	3.01 ± 0.09
33	Spain	Rio Vaca - RVa	2017	9	19.1 ± 0.6	0.026 ± 0.003	2.48 ± 1.38	2.17 ± 0.12
34	Spain	Lebrija – Leb	2017	11	19.8 ± 0.3	0.024 ± 0.001	3.55 ± 1.12	2.95 ± 0.16
22	Spain	Guadalquivir – Gdq	2017	11	19.7 ± 0.5	0.026 ± 0.002	4.31 ± 1.18	2.52 ± 0.17
35	Spain	Doñana N – DoN	2017	9	17.8 ± 0.3	0.022 ± 0.002	2.51 ± 1.14	4.64 ± 0.21
36	Spain	Doñana W – DoW	2017	21	18.4 ± 0.3	0.020 ± 0.002	3.06 ± 0.90	1.98 ± 0.14

Table B.7. Descriptive statistics (mean ± s.e.m.) of life-history traits of wild-caught female *G. holbrooki*.

Site number		Population name and code	Year	*N*	SL [mm]	Lean weight [g]	Fat content [%]	Fecundity	Embryo fat content [%]	Embryo lean weight [mg]	RA [%]
1	Italy	Lago di Garda – LdG	2013	23	28.2 ± 0.4	0.099 ± 0.004	0.59 ± 0.05	35.74 ± 3.20	0.95 ± 0.21	0.66 ± 0.03	18.78 ± 1.28
2	Italy	Comacchio – Com	2013	17	35.5 ± 0.7	0.214 ± 0.015	0.68 ± 0.10	57.41 ± 3.49	0.68 ± 0.18	0.82 ± 0.03	18.99 ± 0.98
3	Italy	Lago di Bolsena – LdB	2013	21	34.0 ± 1.0	0.167 ± 0.017	0.42 ± 0.10	39.86 ± 2.29	1.63 ± 0.32	0.87 ± 0.04	18.48 ± 1.28
4	Italy	Barletta – Blt	2013	18	26.0 ± 0.6	0.069 ± 0.005	2.47 ± 0.33	14.22 ± 1.22	1.88 ± 0.28	0.84 ± 0.07	14.57 ± 1.37
5	Italy	Torre Castiglione – TCa	2013	20	30.2 ± 0.5	0.122 ± 0.008	0.36 ± 0.07	12.55 ± 1.30	1.72 ± 0.64	1.20 ± 0.05	11.92 ± 1.62
6	Italy	Porto Cesareo – PCe	2013	18	27.4 ± 0.4	0.081 ± 0.004	0.61 ± 0.09	15.33 ± 1.53	0.78 ± 0.34	1.12 ± 0.03	17.12 ± 0.89
7	France	Briere – Bri	2013	14	23.9 ± 0.5	0.053 ± 0.004	0.13 ± 0.06	11.50 ± 1.17	0.45 ± 0.21	0.80 ± 0.04	14.97 ± 1.24
8	France	La Ligneron – LLi	2013	21	27.4 ± 0.6	0.088 ± 0.006	0.42 ± 0.09	24.90 ± 2.67	1.18 ± 0.32	0.79 ± 0.02	17.71 ± 0.77
9	France	La Charente – LCh	2013	25	27.9 ± 0.9	0.082 ± 0.010	2.75 ± 0.16	23.84 ± 4.46	0.86 ± 0.15	0.64 ± 0.02	14.33 ± 0.82
10	France	Garonne – Gar	2013	25	26.2 ± 0.4	0.066 ± 0.003	2.38 ± 0.14	28.00 ± 4.71	0.93 ± 0.10	0.64 ± 0.02	19.74 ± 0.93
11	France	Avignon – Avi	2013	23	32.7 ± 0.4	0.152 ± 0.007	1.21 ± 0.09	89.04 ± 5.62	0.78 ± 0.16	0.49 ± 0.02	21.90 ± 0.82
12	France	Arles – Arl	2013	25	35.4 ± 0.7	0.164 ± 0.009	2.13 ± 0.19	39.44 ± 3.29	2.08 ± 0.48	1.00 ± 0.05	18.46 ± 0.70
13	France	Montpellier – Mon	2013	18	26.5 ± 1.1	0.063 ± 0.005	0.31 ± 0.10	20.83 ± 1.70	0.58 ± 0.23	0.70 ± 0.04	18.87 ± 1.26
14	Spain	Zadorra – Zad	2013	14	26.3 ± 0.4	0.076 ± 0.002	0.44 ± 0.09	33.86 ± 2.57	0.78 ± 0.27	0.52 ± 0.02	18.76 ± 0.96
15	Spain	Rio Ter – RTe	2013	15	27.9 ± 0.6	0.079 ± 0.005	1.44 ± 0.17	20.13 ± 2.77	0.45 ± 0.10	0.90 ± 0.04	17.59 ± 1.15
16	Spain	Barcelona – Bcn	2013	20	26.8 ± 0.6	0.076 ± 0.005	0.49 ± 0.10	14.70 ± 1.94	0.43 ± 0.10	1.02 ± 0.03	15.95 ± 1.41
17	Spain	Ebro Delta – EDe	2013	21	31.9 ± 0.7	0.133 ± 0.011	3.86 ± 0.75	54.95 ± 4.36	7.41 ± 0.69	0.68 ± 0.03	22.57 ± 0.79
18	Spain	Sagunt – Sag	2013	22	29.5 ± 0.8	0.104 ± 0.010	0.72 ± 0.11	17.82 ± 1.45	0.57 ± 0.10	0.92 ± 0.06	13.80 ± 0.87
19	Spain	Rio Júcar S – RJS	2013	24	33.5 ± 0.5	0.140 ± 0.008	1.90 ± 0.36	36.92 ± 2.73	1.33 ± 0.29	0.99 ± 0.02	20.30 ± 0.68
20	Spain	Rio Segura – RSe	2013	25	23.2 ± 0.4	0.047 ± 0.003	0.69 ± 0.09	11.04 ± 1.27	0.59 ± 0.11	0.86 ± 0.04	15.60 ± 1.14
21	Spain	Almanzora – Alm	2013	18	26.2 ± 0.7	0.074 ± 0.007	0.70 ± 0.11	9.28 ± 1.05	1.31 ± 0.27	0.98 ± 0.04	11.66 ± 1.28
22	Spain	Guadalquivir – Gdq	2013	22	29.9 ± 0.9	0.110 ± 0.010	0.28 ± 0.07	18.68 ± 1.46	0.29 ± 0.09	0.97 ± 0.03	14.59 ± 0.88
23	Spain	Guadiaro – Gdr	2013	23	31.9 ± 1.0	0.127 ± 0.010	0.46 ± 0.08	34.13 ± 3.87	1.67 ± 0.41	0.83 ± 0.05	17.73 ± 1.12

Table B.7 continued.

24	Italy	Lago di Fimon W – LFW	2017	29	27.5 ± 1.0	0.092 ± 0.011	2.35 ± 0.33	16.45 ± 2.35	5.78 ± 0.92	0.84 ± 0.03	13.41 ± 0.83
25	Italy	Lago di Fimon S – LFS	2017	10	30.0 ± 1.8	0.120 ± 0.022	2.29 ± 0.45	24.20 ± 2.28	3.24 ± 1.08	0.76 ± 0.03	14.73 ± 0.87
26	Italy	Marina Grosseto – MGr	2017	20	24.5 ± 1.1	0.063 ± 0.009	3.43 ± 0.69	23.35 ± 3.29	3.63 ± 1.01	0.83 ± 0.03	23.52 ± 1.28
27	Italy	Grosseto – Gro	2017	10	25.6 ± 1.1	0.070 ± 0.008	2.49 ± 0.54	27.90 ± 5.10	0.72 ± 0.41	0.55 ± 0.01	16.67 ± 1.28
5	Italy	Torre Castiglione – TCa	2017	27	30.9 ± 0.5	0.132 ± 0.008	1.46 ± 0.28	33.37 ± 2.00	2.13 ± 0.55	1.11 ± 0.04	22.43 ± 1.11
6	Italy	Porto Cesareo – PCe	2017	7	20.6 ± 0.3	0.037 ± 0.002	2.07 ± 1.07	5.86 ± 0.40	0.63 ± 0.43	0.83 ± 0.08	11.81 ± 1.45
28	Spain	Gualta – Glt	2017	24	24.4 ± 0.6	0.059 ± 0.005	2.63 ± 0.46	14.54 ± 1.40	4.97 ± 0.86	0.82 ± 0.03	17.25 ± 0.94
29	Spain	Tortosa – Tor	2017	12	26.9 ± 0.8	0.080 ± 0.008	2.88 ± 0.41	26.5 ± 3.97	1.57 ± 0.47	0.75 ± 0.03	19.05 ± 1.89
30	Spain	L'Estanyol – Est	2017	0	-	-	-	-	-	-	-
18	Spain	Sagunt – Sag	2017	6	26.7 ± 2.1	0.081 ± 0.022	1.53 ± 0.57	14.17 ± 4.33	2.26 ± 1.46	1.02 ± 0.06	14.77 ± 1.20
31	Spain	El Palmar – EPa	2017	28	29.6 ± 0.6	0.113 ± 0.007	1.64 ± 0.24	43.07 ± 5.78	1.27 ± 0.22	0.70 ± 0.03	18.63 ± 1.74
32	Spain	Rio Júcar N – RJN	2017	8	35.8 ± 1.0	0.196 ± 0.019	1.43 ± 0.23	60.00 ± 8.09	0.30 ± 0.16	0.75 ± 0.04	17.88 ± 1.84
33	Spain	Rio Vaca – RVa	2017	9	24.2 ± 1.9	0.060 ± 0.014	2.12 ± 0.73	8.89 ± 2.53	2.88 ± 1.42	0.72 ± 0.04	9.49 ± 1.07
34	Spain	Lebrija – Leb	2017	10	28.1 ± 0.9	0.085 ± 0.008	1.78 ± 0.41	14.90 ± 1.93	0.94 ± 0.23	1.16 ± 0.03	16.67 ± 1.02
22	Spain	Guadalquivir – Gdq	2017	10	29.4 ± 0.6	0.105 ± 0.006	0.53 ± 0.24	19.60 ± 1.63	1.67 ± 0.49	1.15 ± 0.04	17.68 ± 0.96
35	Spain	Doñana N – DoN	2017	10	30.8 ± 1.1	0.127 ± 0.015	0.50 ± 0.22	58.10 ± 11.26	2.74 ± 1.53	0.56 ± 0.05	19.31 ± 2.17
36	Spain	Doñana W – DoW	2017	19	28.9 ± 0.8	0.103 ± 0.009	5.90 ± 1.04	12.58 ± 1.88	7.58 ± 1.43	1.10 ± 0.07	11.58 ± 1.24

Table B.8. MANCOVA on phenotypic variation in invasive mosquitofish between different populations and population genetic clusters. We inferred population genetic cluster information for our Italian and Spanish populations sampled in 2017 from the STRUCTURE analysis of our 2013 samples (Fig. 3.2*b*) for *K* = 4. (*a*) Male and female body shape, (*b*) male and (*c*) female life-history traits. Significant effects are highlighted in bold.

	Factor	*F*	Degrees of freedom	*P*	Partial η^2
(*a*) male and female body shape	**Sex**	8,842.644	3, 1252	**< 0.001**	0.955
	Year	17.112	3, 1252	**< 0.001**	0.039
	Centroid size	8.630	3, 1252	**< 0.001**	0.020
	Cluster	11.347	3, 1252	**< 0.001**	0.026
	Population(cluster)	7.829	102, 3750	**< 0.001**	0.175
	Sex × year	2.169	3, 1252	0.090	0.005
	Sex × population (cluster)	4.754	102, 3750	**< 0.001**	0.114
	Year × cluster	5.626	3, 1252	**0.001**	0.013
	Year × population (cluster)	5.414	6, 2504	**< 0.001**	0.013
(*b*) male life-history traits	**SL**	510.221	3, 544	**< 0.001**	0.738
	Year	12.931	3, 544	**< 0.001**	0.067
	Cluster	3.152	3, 544	**0.025**	0.017
	Population(cluster)	4.522	102, 1639	**< 0.001**	0.220
	SL × population (cluster)	1.347	105, 1630	**0.013**	0.080
	Year × cluster	2.688	3, 544	**0.046**	0.015
(*c*) female life-history traits	**Embryo stage**	7.081	6, 633	**< 0.001**	0.063
	SL	655.886	6, 633	**< 0.001**	0.861
	Year	8.453	6, 633	**< 0.001**	0.074
	Cluster	14.974	6, 633	**< 0.001**	0.124
	Population(cluster)	7.698	198, 3753	**< 0.001**	0.265
	SL × cluster	5.040	6, 633	**< 0.001**	0.046
	SL × population (cluster)	3.108	198, 3753	**< 0.001**	0.139
	Year × cluster	7.763	6, 633	**< 0.001**	0.069

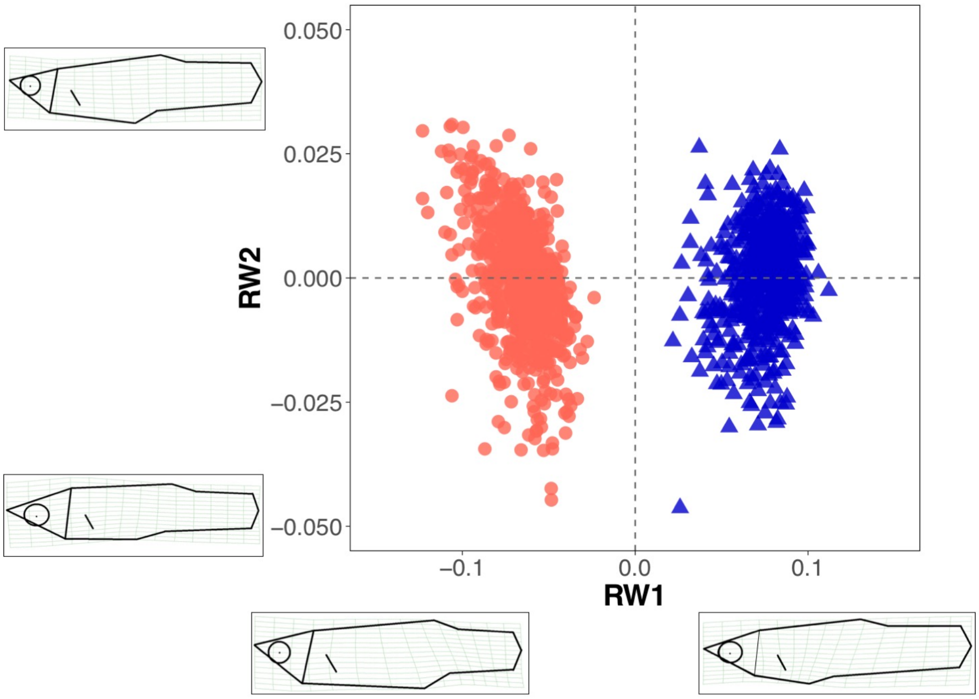

Figure B.3. Sexual dimorphism in body shape in wild-caught *G. holbrooki*. Males: blue triangles, females: red circles. Morphological variation is visualised using thin-plate spline transformation. RW1 mainly separates males (anal fin positioned towards the anterior, creating a small body cavity) from females (anal fin positioned towards the posterior, creating a large body cavity), whereas RW2 describes differences in body depth and head size.

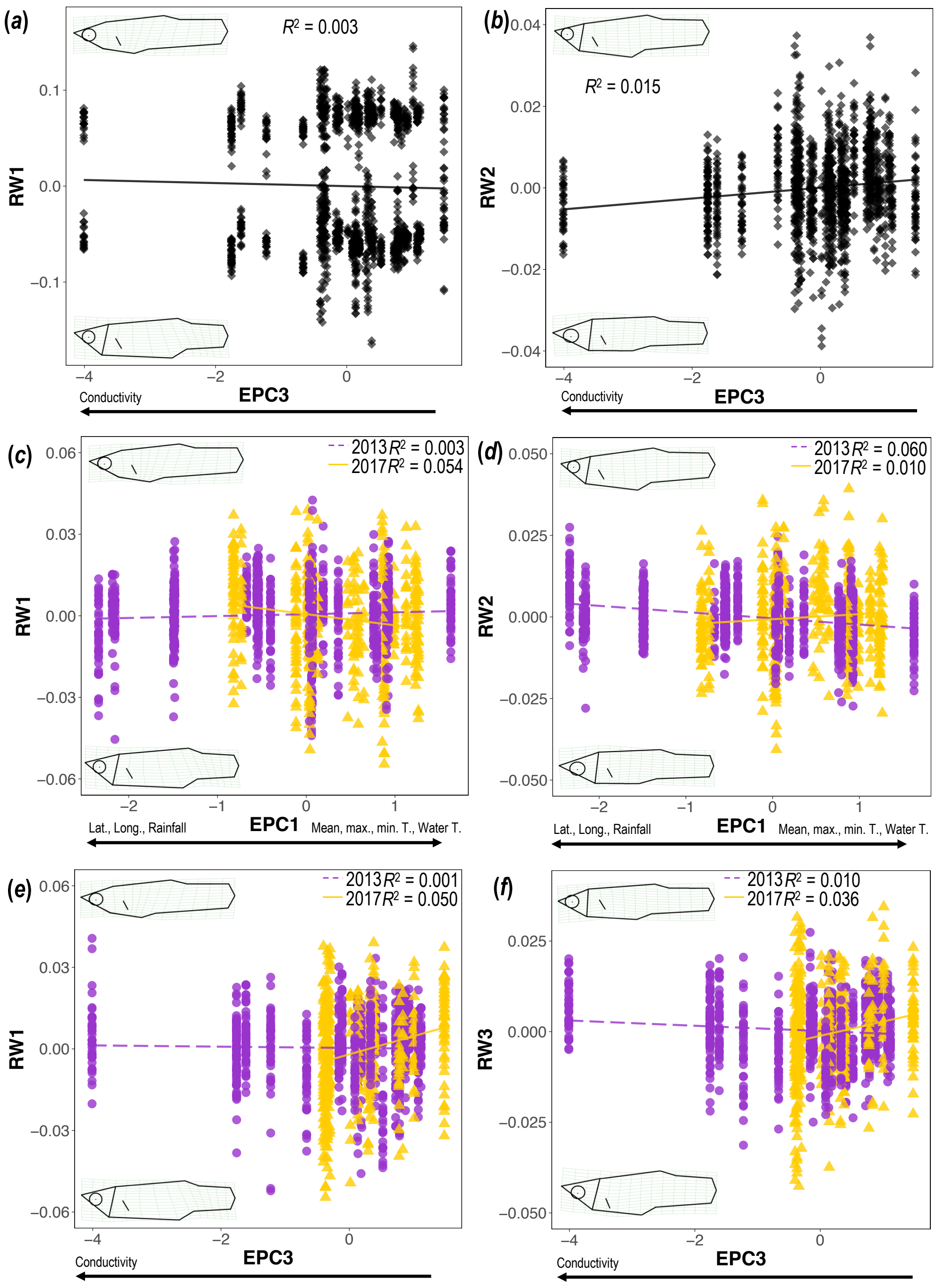

Figure B.4. Partial regression plots of climate-dependent (EPC1) and -independent (EPC3) variation of body shape in wild-caught *G. holbrooki*. The plots (and regression lines) are derived from ANCOVAs and data points represent residuals corrected for all the other terms in the models. (*a*) RW1, (*b*) RW2 variation along EPC3; (*c*) RW1 and (d) RW2 variation along EPC1; (*e*) RW1 and (*f*) RW2 variation across EPC3 between fish sampled in 2013 (purple circles) and 2017 (yellow triangles).

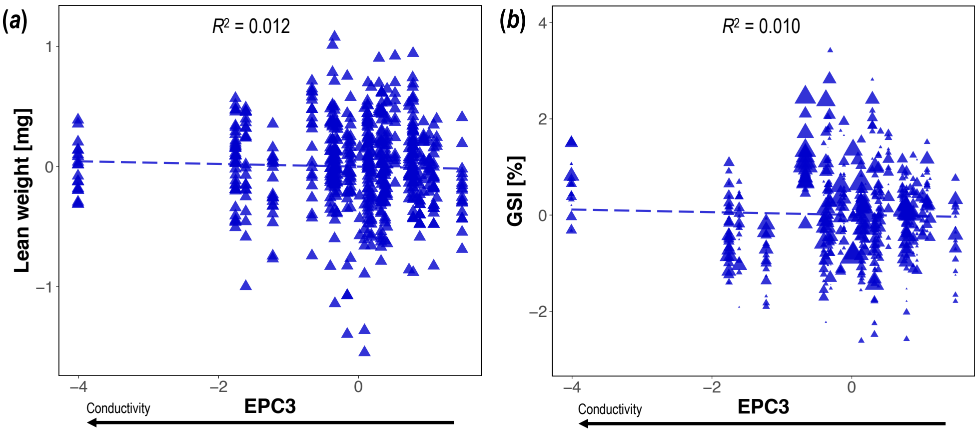

Figure B.5. Partial regression plots of life-history variation along EPC3 in male wild-caught *G. holbrooki*. The plots (and regression lines) are derived from ANCOVAs, and data points represent residuals corrected for all the other terms in the models. (*a*) Lean weight, and (*b*) GSI variation. In (*b*), the size of the data points is proportional to SL.

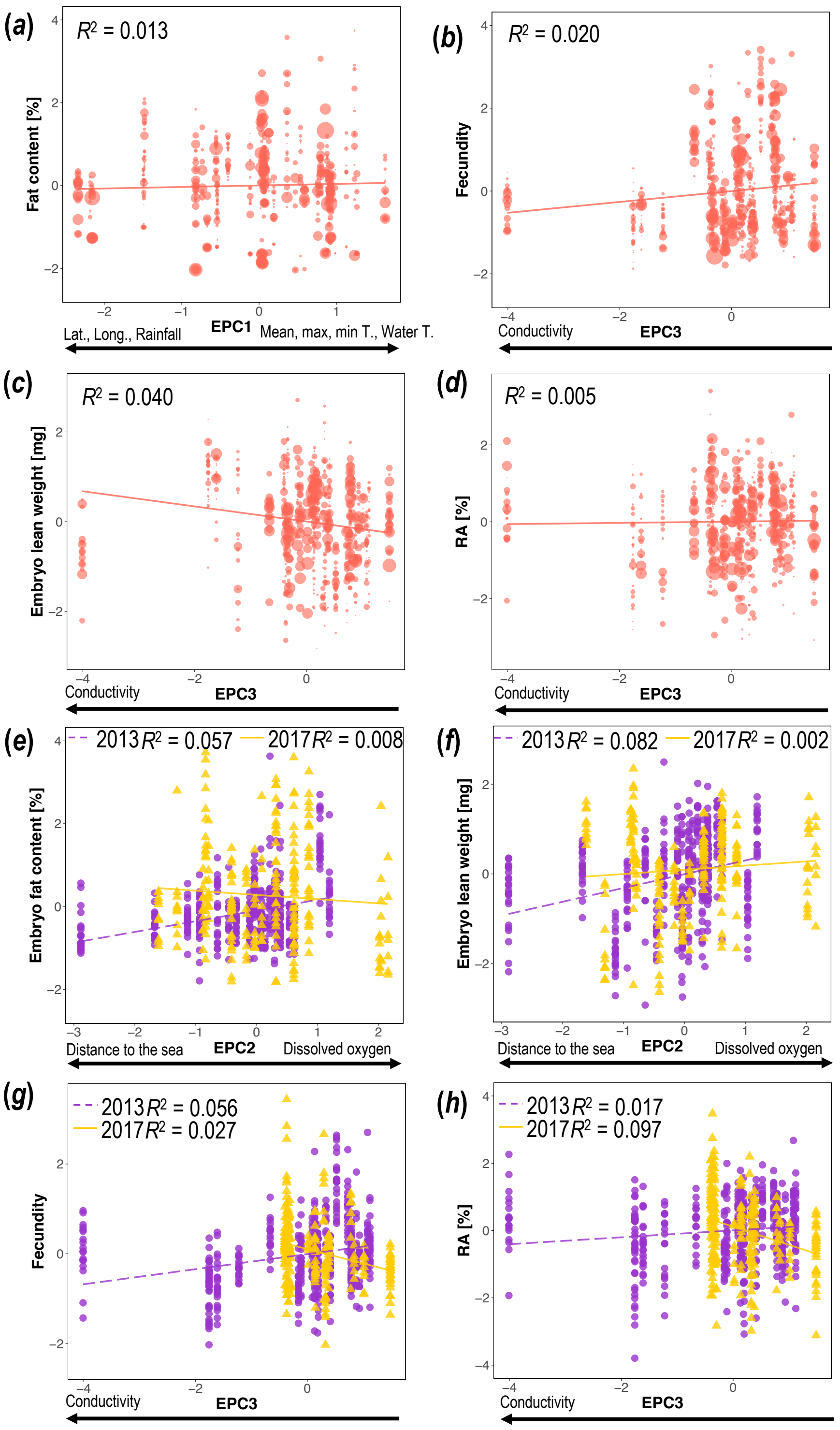

Figure B.6. Partial regression plots of climate-dependent (EPC1) and -independent (EPC2, EPC3) life-history variation of female wild-caught *G. holbrooki*. The plots (and regression lines) are derived from ANCOVAs, and data points represent residuals corrected for all the other terms in the models. (*a*) Fat content variation along EPC1; (*b*) fecundity, (*c*) embryo lean weight, and (*d*) RA variation along EPC3. The size of the data points is proportional to SL. (*e*) Embryo fat content, and (*f*) embryo lean weight variation along EPC2, and (*g*) fecundity, and (*h*) RA variation across EPC3 in fish sampled in 2013 (purple circles) and 2017 (yellow triangles).

Appendix B4: Extended results – phenotypic differences between wild-caught and laboratory-raised mosquitofish

The ANCOVA on male and female SL yielded significant effects of 'sex' ($F_{1, 224} = 469.54$, $P < 0.001$, $\eta_p^2 = 0.689$), 'population' ($F_{3, 224} = 8.51$, $P < 0.001$; $\eta_p^2 = 0.105$), 'generation' ($F_{1, 224} = 138.85$, $P < 0.001$, $\eta_p^2 = 0.396$), but also of the interactions 'population × generation' ($F_{3, 224} = 32.24$, $P < 0.001$, $\eta_p^2 = 0.349$), 'sex × population' ($F_{3, 224} = 9.26$, $P < 0.001$, $\eta_p^2 = 0.129$), and 'sex × population × generation' ($F_{4, 224} = 11.08$, $P < 0.001$, $\eta_p^2 = 0.249$). Males (estimated marginal mean ± s.e.m. = 23.16 ± 0.29 mm) were smaller than females (32.31 ± 0.29 mm), and overall, the largest fish came from Lago di Garda (29.32 ± 0.48 mm), with intermediate-sized fish at Comacchio (28.20 ± 0.40 mm) and Torre Castiglione (27.14 ± 0.32 mm), while the smallest fish originated from Zadorra (26.29 ± 0.43 mm). However, the smallest males came from Zadorra (21.26 ± 0.59 mm) and the largest ones from Torre Castiglione (24.10 ± 0.45 mm) and Comacchio (24.09 ± 0.55 mm), whereas the smallest females came from Torre Castiglione (30.18 ± 0.45 mm) and by far the largest females from Lago di Garda (35.44 ± 0.66 mm). In addition, second-generation laboratory-reared fish were larger (30.23 ± 0.32 mm) than wild-caught individuals (25.25 ± 0.26 mm), but this size difference was population-specific, with the largest increase in fish from Lago di Garda (ΔSL = 9.37 mm), followed by fish from Zadorra (ΔSL = 8.14 mm) and Torre Castiglione (ΔSL = 4.33 mm), while fish from Comacchio actually became smaller (ΔSL = 2.28 mm). For males, the relative size differences between wild-caught males from different populations were largely replicated for F_2 laboratory-reared males, but population-specific patterns of female size differed between wild-caught and laboratory-reared females (Fig. 3.4*a*).

The MANCOVA on body shape revealed significant effects of the covariate 'centroid size' ($F_{3, 242} = 10.28$, $P < 0.001$) but also of all factors and interactions (sex: $F_{3, 242} = 1{,}890.81$, $P < 0.001$; population: $F_{9, 589} = 3.02$, $P = 0.002$; generation: $F_{3, 242} = 13.74$, $P < 0.001$; sex × generation: $F_{3, 242} = 5.09$,

$P = 0.002$; sex × population: $F_{9,589} = 2.80$, $P = 0.003$; population × generation: $F_{9,589} = 6.85$, $P < 0.001$; sex × population × generation: $F_{9,589} = 3.14$, $P = 0.001$). 'Sex' had by far the strongest effect ($\eta_p^2 = 0.959$), followed by 'generation' ($\eta_p^2 = 0.146$) and 'centroid size' ($\eta_p^2 = 0.113$), while 'population' and all interactions were of relatively minor importance ($\eta_p^2 < 0.079$ in all cases). *Post-hoc* ANCOVAs ($\alpha' = 0.017$, corrected for multiple testing) revealed that sexes differed along RW1 ($F_{1,244} = 5,070.23$, $P < 0.001$, $\eta_p^2 = 0.954$) with males being characterized by larger eyes, smaller heads, deeper caudal peduncles, and an anal fin that was positioned more anteriorly compared to females. Populations differed significantly in RW3 ($F_{3,244} = 1.07$, $P = 0.001$, $\eta_p^2 = 0.065$), with fish from Zadorra having slightly deeper bodies, smaller eyes, a slightly more superiorly-positioned mouth, slightly more ventrally-positioned pectoral fins, and the dorsal part of their heads extending further into the body compared to fish from Torre Castiglione, while fish from Comacchio and Lago di Garda showed intermediate character state combinations. Lastly, wild-caught and laboratory-reared fish differed significantly in RW1 ($F_{1,244} = 21.67$, $P < 0.001$, $\eta_p^2 = 0.082$) and RW2 ($F_{1,244} = 20.24$, $P < 0.001$, $\eta_p^2 = 0.077$). Wild-caught fish had slightly deeper bodies, shorter caudal peduncles, smaller eyes, and their anal fin was positioned more posteriorly compared to laboratory-reared individuals (RW1), and showed slightly deeper bodies, larger heads coupled with larger eyes, and more posteriorly-positioned pectoral fins (RW2). However, the interaction effects indicated that the differences between populations were both sex-specific and moderated by differences between wild-caught and laboratory reared fish.

The MANCOVA on male life histories revealed significant effects of the covariate SL ($F_{3,111} = 207.68$, $P < 0.001$, $\eta_p^2 = 0.849$), the factors 'population' ($F_{9,270} = 6.02$, $P < 0.001$, $\eta_p^2 = 0.138$) and 'generation' ($F_{3,111} = 87.80$, $P < 0.001$, $\eta_p^2 = 0.704$), as well as the interaction 'population × generation' ($F_{9,270} = 11.44$, $P < 0.001$, $\eta_p^2 = 0.230$). *Post-hoc* ANCOVAs ($\alpha' = 0.017$) of the same model structure indicated that SL had significant effects on all three traits ($P < 0.001$ in all cases), and both lean weight ($\eta_p^2 = 0.848$) and body fat ($\eta_p^2 = 0.327$) increased with increasing SL, while GSI ($\eta_p^2 =$

0.142) decreased. Populations differed significantly in lean weight (P = 0.007, $\eta_{\mathrm{p}}^2 = 0.102$) and fat content ($P < 0.001$, $\eta_{\mathrm{p}}^2 = 0.193$), with males from Torre Castiglione having the smallest and males from Zadorra the greatest lean weight (Fig 3.4*b*; Table B.9). Wild-caught fish differed from F_2 laboratory-reared fish in all three traits ($P < 0.001$ in all cases), with lean weight ($\eta_{\mathrm{p}}^2 = 0.245$) and GSI ($\eta_{\mathrm{p}}^2 = 0.225$) being lower in laboratory-reared fish and fat content ($\eta_{\mathrm{p}}^2 = 0.575$) being higher (Table B.10). Nonetheless, there were also population-specific responses to the laboratory environment in fat content ($\eta_{\mathrm{p}}^2 = 0.314$) and GSI ($\eta_{\mathrm{p}}^2 = 0.436$; $P < 0.001$ in both cases), while this was not evident for lean weight ($P = 0.889$). Fat content increased in all populations, but showed by far the strongest increase in Comacchio (Δfat content = 18.60%) and the weakest increase in Zadorra (Δfat content = 3.10%; Table S7). GSI, on the other hand, increased from wild-caught to laboratory-raised males only in Zadorra, while it decreased in all other populations (Table B.11).

The MANCOVA on female life histories demonstrated significant effects of the covariates ‘embryonic stage of development’ ($F_{6,\,103} = 2.26$, P = 0.043, $\eta_{\mathrm{p}}^2 = 0.116$) and SL ($F_{6,\,103} = 40.03$, $P < 0.001$, $\eta_{\mathrm{p}}^2 = 0.700$), the factors ‘population’ ($F_{18,\,292} = 12.06$, $P < 0.001$, $\eta_p^2 = 0.408$) and ‘generation’ ($F_{6,\,103} = 127.39$, $P < 0.001$, $\eta_p^2 = 0.881$), as well as the interaction ‘population × generation’ ($F_{18,\,292} = 6.97$, $P < 0.001$, $\eta_{\mathrm{p}}^2 = 0.286$). *Post-hoc* ANCOVAs ($\alpha' = 0.008$) of the same model structure revealed that ‘embryonic stage of development’ did not significantly affect any individual life-history trait, while SL had significant effects on lean weight ($\eta_{\mathrm{p}}^2 = 0.652$), fat content ($\eta_{\mathrm{p}}^2 = 0.140$), fecundity ($\eta_{\mathrm{p}}^2 = 0.234$) and embryo fat ($\eta_{\mathrm{p}}^2 = 0.108$; $P < 0.001$ in all cases), all of which increased with increasing female size. Populations differed significantly in fat content ($\eta_{\mathrm{p}}^2 = 0.404$), fecundity ($\eta_{\mathrm{p}}^2 = 0.377$), embryo lean weight ($\eta_{\mathrm{p}}^2 = 0.314$; $P < 0.001$ in all cases), and embryo fat content ($P = 0.001$, $\eta_{\mathrm{p}}^2 = 0.144$; Fig. 3.4*c*). Females from Zadorra had the greatest somatic fat content and also had embryos with the greatest fat content, and together with females from Comacchio, had the highest fecundity (Table B.12). Females from Torre Castiglione had the lowest fat content and fecundity, coupled with the heaviest embryos, and females from

Comacchio had embryos with the lowest fat content (Table B.12). Wild-caught females differed from F_2 laboratory-reared females in somatic fat content ($\eta_p^2 = 0.861$), embryo lean weight ($\eta_p^2 = 0.538$), embryo fat content ($\eta_p^2 = 0.626$; all $P < 0.001$), and fecundity ($P = 0.001$, $\eta_p^2 = 0.092$), with somatic fat content, embryo lean weight and embryo fat content being higher in laboratory-reared fish and fecundity being higher in wild-caught fish (Table B.13). Furthermore, there were again population-specific responses to the laboratory environment. These were uncovered for fat content ($\eta_p^2 = 0.428$), fecundity ($\eta_p^2 = 0.268$), embryo lean weight ($\eta_p^2 = 0.334$), and RA ($\eta_p^2 = 0.179$; $P < 0.001$ in all cases). Fat content increased in all populations, but showed the strongest increase in Comacchio (Δfat content = 29.4%) and the weakest increase in Torre Castiglione (Δfat content = 10.9%; Table B.14). Fecundity, on the other hand, decreased from wild-caught to laboratory-raised fish in Zadorra, Comacchio and Lago di Garda, while it increased in Torre Castiglione (Table B.14). Embryo lean weight increased in all populations from wild-caught to lab-raised females, with the strongest increase in Lago di Garda females (Δembryo lean weight = 0.880 mg) and the weakest increase in females from Torre Castiglione (Δembryo lean weight = 0.256 mg). Lastly, RA strongly increased from wild-caught to lab-raised females in Torre Castiglione (ΔRA = 9.3%), remained rather unchanged in both Zadorra (ΔRA = 0.2%) and Lago di Garda (ΔRA = 0.5%), but strongly decreased in Comacchio (ΔRA = 4.6%; Table B14).

Table B.9. Estimated marginal means and standard errors (s.e.m.) for significant population-level differences in male lean weight and somatic fat content (derived from MANCOVA; evaluated at SL = 23.17 mm).

Trait	Population	Mean	s.e.m.
Lean weight [mg]	Zadorra (ES)	52.26	1.84
	Torre Castiglione (IT)	43.93	1.34
	Comacchio (IT)	45.69	1.62
	Lago di Garda (IT)	49.42	2.07
Fat content [%]	Zadorra (ES)	8.55	0.90
	Torre Castiglione (IT)	4.72	0.66
	Comacchio (IT)	10.03	0.80
	Lago di Garda (IT)	8.96	1.01

Table B.10. Estimated marginal means and standard errors (s.e.m.) for significant differences in lean weight, fat content and GSI between wild-caught and second-generation laboratory-reared male *G. holbrooki* (derived from MANCOVA; evaluated at SL = 23.17 mm).

Trait	Generation	Mean	s.e.m.
Lean weight [mg]	Wild-caught	55.51	1.40
	Lab-reared	41.14	1.37
Fat content [%]	Wild-caught	3.48	0.69
	Lab-reared	12.64	0.67
GSI [%]	Wild-caught	2.29	0.09
	Lab-reared	1.27	0.09

Table B.11. Estimated marginal means and standard errors (s.e.m.) for significant population-by-generation differences in male fat content and GSI (derived from MANCOVA; evaluated at SL = 23.17 mm).

Trait	Population	Generation	Mean	s.e.m.
Fat content [%]	Zadorra (ES)	Wild-caught	6.99	1.18
		Lab-reared	10.10	1.43
	Torre Castiglione (IT)	Wild-caught	3.04	0.94
		Lab-reared	6.39	1.08
	Comacchio (IT)	Wild-caught	0.73	0.90
		Lab-reared	19.32	1.31
	Lago di Garda (IT)	Wild-caught	3.16	1.84
		Lab-reared	14.76	0.99
GSI [%]	Zadorra (ES)	Wild-caught	1.77	0.16
		Lab-reared	2.39	0.20
	Torre Castiglione (IT)	Wild-caught	1.78	0.13
		Lab-reared	1.28	0.15
	Comacchio (IT)	Wild-caught	3.18	0.12
		Lab-reared	0.69	0.18
	Lago di Garda (IT)	Wild-caught	2.41	0.25
		Lab-reared	0.74	0.13

Table B.12. Estimated marginal means and standard errors (s.e.m.) for significant population-level differences in female fat content, fecundity, embryo lean weight, and embryo fat content (derived from MANCOVA; evaluated at SL = 30.78 mm and an embryonic stage of development = 15.38).

Trait	Population	Mean	s.e.m.
Fat content [%]	Zadorra (ES)	16.07	0.73
	Torre Castiglione (IT)	7.35	0.50
	Comacchio (IT)	13.83	0.66
	Lago di Garda (IT)	13.21	0.95
Fecundity	Zadorra (ES)	34.31	2.56
	Torre Castiglione (IT)	17.07	1.77
	Comacchio (IT)	34.97	2.32
	Lago di Garda (IT)	32.30	3.35
Embryo lean weight [mg]	Zadorra (ES)	0.99	0.05
	Torre Castiglione (IT)	1.31	0.03
	Comacchio (IT)	1.15	0.04
	Lago di Garda (IT)	1.12	0.06
Embryo fat content [%]	Zadorra (ES)	10.59	0.68
	Torre Castiglione (IT)	7.67	0.47
	Comacchio (IT)	5.72	0.61
	Lago di Garda (IT)	6.85	0.88

Table B.13. Estimated marginal means and standard errors (s.e.m.) for significant differences in female fat content, fecundity, embryo lean weight and embryo fat content between wild-caught and second-generation laboratory-reared *G. holbrooki* (derived from MANCOVA; evaluated at SL = 30.78 mm and an embryonic stage of development = 15.38).

Trait	Generation	Mean	s.e.m.
Fat content [%]	Wild-caught	1.68	0.42
	Lab-reared	23.54	0.74
Fecundity	Wild-caught	35.18	1.47
	Lab-reared	24.15	2.58
Embryo lean weight [mg]	Wild-caught	0.82	0.03
	Lab-reared	1.47	0.05
Embryo fat content [%]	Wild-caught	1.74	0.39
	Lab-reared	13.68	0.68

Table B.14. Estimated marginal means and standard errors (s.e.m.) for significant population-by-generation differences in female fat content, fecundity, embryo lean weight and RA (derived from MANCOVA; evaluated at SL = 30.78 mm and embryonic stage of development = 15.38).

Trait	Population	Generation	Mean	s.e.m.
Fat content [%]	Zadorra (ES)	Wild-caught	4.08	0.96
		Lab-reared	27.99	1.28
	Torre Castiglione (IT)	Wild-caught	1.91	0.72
		Lab-reared	12.77	0.74
	Comacchio (IT)	Wild-caught	< 0.01	0.92
		Lab-reared	28.54	1.07
	Lago di Garda (IT)	Wild-caught	1.55	0.79
		Lab-reared	24.85	1.80
Fecundity	Zadorra (ES)	Wild-caught	38.43	3.39
		Lab-reared	29.75	4.52
	Torre Castiglione (IT)	Wild-caught	14.22	2.54
		Lab-reared	19.95	2.61
	Comacchio (IT)	Wild-caught	49.44	3.22
		Lab-reared	20.20	3.78
	Lago di Garda (IT)	Wild-caught	37.85	2.79
		Lab-reared	26.92	6.35
Embryo lean weight [mg]	Zadorra (ES)	Wild-caught	0.63	0.06
		Lab-reared	1.34	0.08
	Torre Castiglione (IT)	Wild-caught	1.19	0.05
		Lab-reared	1.44	0.05
	Comacchio (IT)	Wild-caught	0.76	0.06
		Lab-reared	1.51	0.07
	Lago di Garda (IT)	Wild-caught	0.68	0.05
		Lab-reared	1.56	0.12
RA [%]	Zadorra (ES)	Wild-caught	18.78	1.81
		Lab-reared	18.85	2.42
	Torre Castiglione (IT)	Wild-caught	10.77	1.36
		Lab-reared	20.06	1.40
	Comacchio (IT)	Wild-caught	20.58	1.72
		Lab-reared	15.87	2.02
	Lago di Garda (IT)	Wild-caught	16.89	1.50
		Lab-reared	17.38	3.40

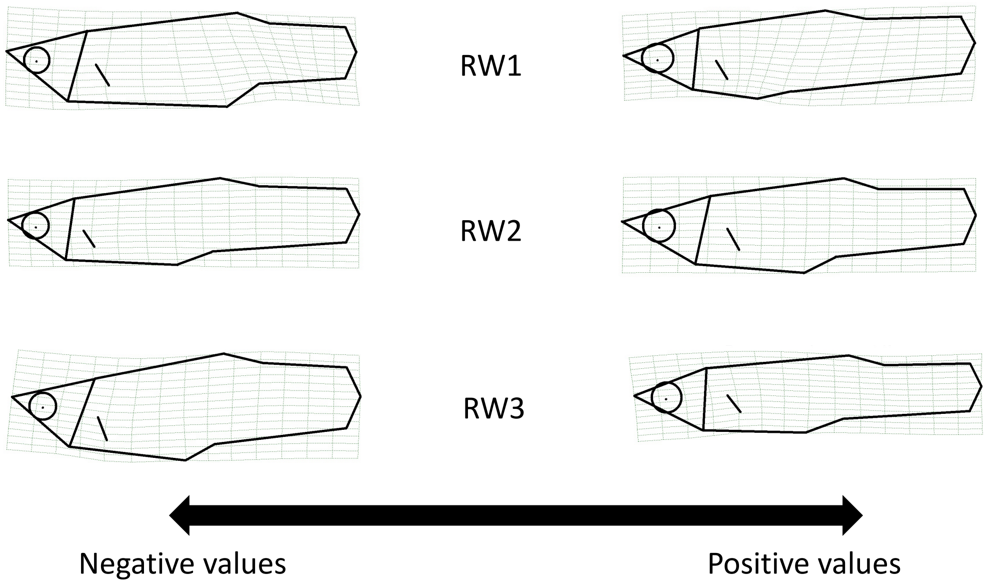

Figure B.7. Thin-plate transformation grids visualising variation in body shape between wild-caught and laboratory-reared mosquitofish. RW1 mainly described differences between males and females, which were mostly due to the anteriorly-shifted anal fin—modified into the gonopodium—in males. RW2 described differences in body depth and head size, while RW3 described differences in both body depth and caudal peduncle depth.

Appendix B5: Additional discussion – yearly variation in life histories and body shape

While some responses to climate variation (and dissolved oxygen) were consistent across both sampling years, several traits showed pronounced inter-annual differences, and their responses to climate gradients were sometimes opposite between 2013 and 2017. These results point towards the presence of plastic responses to selective pressures that we did not quantify with our sampling protocol, but may have differed between sampling years. These could include habitat productivity and food availability (Vondraceck et al. 1988), population density (Bisazza and Marin 1995, Smith and Sargent 2006, Reznick et al. 2019), or parasite load (Benejam et al. 2009).

In particular, both males and females were bigger in 2013 than in 2017. In 2013, males also had reduced fat content and lower GSI, possibly reflecting a trade-off between somatic growth and sperm production (but see Locatello et al. 2008, O'Dea et al. 2014). Additionally, in females, some life-history responses to the latitudinal gradient were reversed between 2013 and 2017. In 2017, they were characterised by increased investment into reproduction in colder environments, congruent with what has been reported for native *G. holbrooki* (Riesch et al. 2018), and with predictions from life-history theory regarding the effects of a high extrinsic mortality in harsh environments (Stearns 1989, Reznick et al. 2002). In 2013, however, these patterns were reversed, as females from southern populations now showed an increased RA. This aligns with results of laboratory studies on the effects of constant temperature differences on *G. affinis* life histories (Vondraceck et al. 1988): higher temperatures caused mosquitofish to mature earlier at bigger body sizes, and to invest more into reproduction. At present, we do not have a convincing explanation for the underlying mechanisms driving these disparate patterns. Nevertheless, our results point towards phenotypic plasticity as a strategy to cope with changing environmental conditions, as demonstrated in other fishes (e.g., *Pomatoschistus microps*, Pampoulie et al. 2000; *Lepomis gibbosus*, Belk 1995; *Gasterosteus aculeatus*, Baker et al. 2015).

Since we sampled our fish at different times throughout the reproductive season in 2013 and 2017, seasonal changes in life-history traits (Hughes 1985) can also play a role in creating the observed phenotypic differences between sampling trips. During the reproductive season, which lasts from early spring to mid-autumn (Fraile et al. 1994, Reznick et al. 2006), mosquitofish increase population sizes (Pyke 2005), which in turn drives body-size and life-history shifts (Hughes 1985, Pyke 2005, Reznick et al. 2006). For example, males born early in the reproductive season reach sexual maturity at smaller body sizes than those born towards the end of the reproductive season (Zulian et al. 1993, Reznick et al. 2006). Small males have higher reproductive success at low population densities, as they are more manoeuvrable and less easily spotted by females during sneaky copulation attempts (Pilastro et al. 1997), while big males have an advantage towards the end of the reproductive season, as they are able to monopolise access to the females (Bisazza and Marin 1995). We accounted for seasonal differences in temperature in our climate data, but we could not properly control for other seasonal differences (e.g., population density). Indeed, inter-annual life-history differences were reduced when Spanish populations (sampled at more or less the same time in 2013 and 2017) were analysed separately, while they were maintained in Italian populations (sampled at the beginning and towards the end of the reproductive season in 2013 and 2017, respectively; results not shown). Future studies that quantify additional environmental selection factors and their seasonal fluctuation are needed in order to properly decipher these mechanisms.

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Appendix C: Supplementary material for Chapter 4 – Geographical and temporal variation of multiple paternity in invasive mosquitofish (*Gambusia holbrooki*, *Gambusia affinis*).

Occurrence of MP in invasive mosquitofish

Several of our microsatellite markers had high polymorphic information contents (PIC) and high combined exclusion probabilities for the second parent (E-2P values; Table C.9), laying a solid base for subsequent analyses of multiple paternity. PIC values ranged from 0.382 to 0.600, and E-2P values were higher than 90% across populations of *G. holbrooki*. Similarly, PIC ranged from 0.477 to 0.717, and E-2P values were above 96% across populations of *G. affinis*. In our monthly sampling of *G. affinis*, we found high PIC and E-2P values in Ankang (PIC: 0.407-0.675, E-2P > 92%; Table C.9*c*), but lower values in Beihai (PIC: 0.282-0.323, E-2P: 76-83%; Table C.9*d*), possibly due to the lower level of genetic variation in the latter population (Table C.10-13). We tested for deviations from Hardy-Weinberg Equilibrium and found only one locus (Gafμ3) to show significant deviations in the Beihai population (Table C.13).

We found a high percentage of multiply sired broods in each of the tested populations (*G. holbrooki*: 40–100%; *G. affinis*: 30–100%; Table C.9). Numbers of sires per brood in *G. holbrooki* varied between 5.00 ± 1.41 (E1, Lago di Fimon C/W) and 1.40 ± 0.55 (E3, Porto Cesareo; Table C.9*a*). Similarly, in *G. affinis*, numbers of sires varied between 3.40 ± 1.35 (C2, Ankang) and 1.40 ± 0.70 (C11, Beihai; Table C.9*b*). Levels of MP in Beihai did not vary much between months (90–100%; Table C.9*d*), while in Ankang both numbers of sires and reproductive skew showed a similar pattern of strong monthly variation (33–100%; Table C.9*c*). MP was highest at the onset of the reproductive season, after which it decreased until it was lowest in July. It increased again until September, and finally sharply decreased in October.

Table C.1. Overview of sampling sites. Number of fish caught (F: females, M: males, J: juveniles) refers to those fish caught in an effort to calculate catch-per-unit-effort (CPUE) and thus does not reflect the sample sizes used for either the MP analyses or for the estimation of population-level life histories.

Group	**Code**	**Location**	**Date**	**Latitude [N]**	**Longitude [E]**	**Number of fish caught (F/M/J)**
(*a*) *G. holbrooki*, geographic variation in MP	E1	Lago di Fimon C/W - IT	2017/7/27	45.47	11.54	—
	E2	Lago di Fimon S - IT	2017/7/27	45.46	11.54	—
	E3	Porto Cesareo - IT	2017/8/3	40.28	17.88	—
	E4	Gualta - ES	2017/8/5	42.03	3.10	—
	E5	El Palmar - ES	2017/8/7	39.31	-0.32	—
	E6	Río Xuquer - ES	2017/8/7	39.18	-0.27	—
	E7	Río Vaca - ES	2017/8/7	39.06	-0.22	—
	E8	Lebrija NE - ES	2017/8/10	36.96	-6.06	—
	E9	Lebrija W- ES	2017/8/10	36.94	-6.10	—
	E10	Doñana - ES	2017/8/10	37.23	-6.14	—
(*b*) *G. affinis*, geographic variation in MP	C1	Baoding	2017/6/7	38.87	115.48	—
	C2	Ankang	2017/4/21	32.73	108.80	—
	C3	Shanghai	2017/4/23	31.11	121.26	—
	C4	Chengdu	2017/6/1	30.57	104.07	—
	C5	Wuhan	2017/4/11	30.46	114.36	—
	C6	Hangzhou	2017/6/8	30.28	120.16	—
	C7	Chongqing	2018/4/11	29.83	106.42	—
	C8	Kunming	2017/4/29	24.99	102.65	—
	C9	Shenzhen	2017/6/2	22.53	114.09	—
	C10	Hepu	2017/4/24	21.64	109.13	—
	C11	Beihai	2017/6/4	21.57	109.14	—
(*c*) *G. affinis*, temporal variation in MP	May	Ankang	2016/5/19	32.73	108.80	67/54/15
	Jun.	Ankang	2016/6/22	32.73	108.80	57/66/34
	Jul.	Ankang	2016/7/21	32.73	108.80	92/55/15
	Aug.	Ankang	2016/8/22	32.73	108.80	97/54/26
	Sept.	Ankang	2016/9/23	32.73	108.80	69/58/18
	Oct.	Ankang	2016/10/22	32.73	108.80	56/48/3
	Apr.	Beihai	2016/4/23	21.57	109.14	185/52/1
	May	Beihai	2016/5/21	21.57	109.14	173/60/30
	Jun.	Beihai	2016/6/25	21.57	109.14	90/34/6
	Jul.	Beihai	2016/7/22	21.57	109.14	122/82/7
	Aug.	Beihai	2016/8/23	21.57	109.14	122/75/17
	Sept.	Beihai	2016/9/21	21.57	109.14	148/36/10

Table C.2. Variation of climatic and geographic parameters across our sampling sites from which invasive mosquitofish were collected. Mean annual temperature, annual temperature difference, max. temperature of the warmest month, min. temperature of the coldest month and annual precipitation were downloaded from Worldclim vs. 2 (http://worldclim.org/version2; Fick and Hijmans 2017). Altitude and distance to the sea were estimated using Google Earth (http://earth.google.com/).

Site code	**Mean annual temp. [°C]**	**Annual temp. difference [°C]**	**Max. temp. of warmest month [°C]**	**Min. temp. of coldest month [°C]**	**Annual precipitation [mm]**	**Altitude [m]**	**Dist. to the sea [km]**
E1	12.7	8.6	26.4	-2.4	983	24	45
E2	12.8	8.8	26.6	-2.5	975	24	44
E3	16.6	7.7	28.5	4.6	515	1	2
E4	15.1	10.0	28.4	3.0	535	6	6
E5	17.3	8.4	29.1	7.1	400	0	1
E6	17.5	9.7	30.3	6.2	382	2	2
E7	17.4	9.7	30.4	5.9	362	3	2
E8	17.9	11.7	30.3	4.3	550	2	33
E9	17.9	11.6	30.1	4.5	552	0	27
E10	17.8	11.7	30.4	3.9	529	3	34
C1	13.2	11.9	32.7	-9.3	537	25	205
C2	15.8	8.9	31.2	-0.7	902	390	1,128
C3	16.3	8.3	32.8	0.5	970	5	54
C4	16.8	7.2	29.4	2.2	987	485	1,682
C5	17.1	8.0	33.3	0.5	1,257	28	607
C6	16.8	7.8	33.0	0.9	1,322	16	52
C7	18.2	6.5	31.0	3.7	1,144	250	965
C8	15.5	7.7	24.8	4.3	911	1,885	650
C9	22.9	6.5	32.6	12.5	1,924	1	8
C10	22.4	6.4	32.8	10.9	1,663	2	8
C11	22.8	6.7	33.1	11.7	1,650	22	2

Table C.3. Results of factor reduction procedures (principal component analysis; PCA) to reduce data dimensionality of (*a*) climatic and geographic variables across sampling sites, (*b*) monthly temperature data for the Ankang and Beihai sampling sites combined. Shown are eigenvalues, total variance explained [%] and axis loadings for different input variables.

Group	Principal components	PC1	PC2	PC3
(*a*) Geographic variation of MP (Europe, China)	Eigenvalue	2.434	1.769	1.687
	Variance explained [%]	34.776	25.274	24.105
	Mean annual temperature	0.888	0.085	0.339
	Annual temperature difference	-0.553	-0.692	0.014
	Max. temperature of warmest month	0.194	0.149	0.904
	Min. temperature of coldest month	0.966	-0.086	-0.036
	Annual precipitation	0.535	0.621	0.339
	Altitude	0.007	0.465	-0.778
	Distance to the sea	-0.286	0.807	-0.185
(*b*) Monthly temperature data for AK and BH	Eigenvalue	3.087	—	—
	Variance explained [%]	77.175	—	—
	Mean max. temperature	0.981	—	—
	Mean min. temperature	0.957	—	—
	Min. temperature of the coldest day	0.941	—	—
	Max. temperature of the warmest day	0.568	—	—

Table C.4. Results of principal component analyses (PCA) on (*a*) population-level life-history traits for the analysis of geographical variation in MP, (*b*) population-level life-history traits of *G. affinis* from Ankang and Beihai for the analysis of temporal variation. Shown are eigenvalues, total variance explained [%] and axis loadings for different input variables.

Group	**Principal components**	**LHPC1**	**LHPC2**	**LHPC3**	**LHPC4**
(*a*) Life-history traits of *G. holbrooki* (Europe) and *G. affinis* (China)	Eigenvalue	3.364	2.759	1.891	1.061
	Variance explained [%]	30.581	25.085	17.194	9.646
	Male SL	0.785	-0.130	0.311	-0.183
	Male lean weight	-0.742	0.257	0.022	0.263
	Male fat content	0.523	0.146	0.498	0.526
	GSI	-0.601	0.649	0.107	0.230
	Females SL	0.408	0.645	0.461	-0.071
	Female lean weight	0.800	0.222	-0.349	-0.332
	Female fat content	0.565	-0.134	-0.527	0.282
	Fecundity	0.129	0.891	-0.346	0.106
	Embryo lean weight	-0.141	-0.569	0.768	-0.067
	Embryo fat content	0.541	-0.347	-0.057	0.639
	RA	0.327	0.716	0.457	-0.099
(*b*) Life-history traits of *G. affinis* from Ankang and Beihai	Eigenvalue	5.369	1.631	1.602	—
	Variance explained [%]	48.813	14.830	14.560	—
	Male SL	0.768	0.113	-0.436	—
	Male lean weight	0.847	0.175	-0.328	—
	Male fat content	0.523	0.545	-0.366	—
	GSI	0.712	0.014	-0.392	—
	Females SL	0.564	-0.190	0.350	—
	Female lean weight	0.788	0.087	-0.194	—
	Female fat content	0.839	-0.253	0.347	—
	Fecundity	0.581	-0.753	-0.190	—
	Embryo lean weight	0.370	0.750	0.470	—
	Embryo fat content	0.688	0.086	0.617	—
	RA	0.830	-0.215	0.312	—

Table C.5. Descriptive statistics (mean ± SD) of female body size (SL), fecundity and estimates of MP for (*a*) European *G. holbrooki*, (*b*) Chinese *G. affinis*, as well as for monthly samples of *G. affinis* from (*c*) Ankang and (*d*) Beihai. Please note that these are not the same females that were used to quantify population-level life histories, which we used in our path analyses.

	Code	**N of mothers**	**SL [mm]**	**Fecundity**	**Number of sires**	**Repr. skew**
(*a*) Geographic variation, *G. holbrooki*	E1	8	25.84 ± 2.84	12.75 ± 2.05	5.00 ± 1.41	0.29 ± 0.15
	E2	8	24.66 ± 8.31	22.50 ± 27.93	4.63 ± 2.45	0.21 ± 0.16
	E3	5	19.10 ± 1.81	3.80 ± 1.30	1.40 ± 0.55	0.05 ± 0.09
	E4	8	22.26 ± 2.72	8.25 ± 1.91	2.63 ± 1.69	0.21 ± 0.20
	E5	10	29.77 ± 2.53	49.20 ± 24.94	3.30 ± 1.16	0.22 ± 0.13
	E6	9	29.91 ± 4.42	28.11 ± 13.87	2.11 ± 0.60	0.17 ± 0.12
	E7	10	23.38 ± 4.45	11.20 ± 8.88	2.60 ± 1.27	0.15 ± 0.17
	E8	9	24.53 ± 1.98	6.22 ± 3.53	2.11 ± 1.05	0.14 ± 0.15
	E9	8	25.60 ± 3.00	9.50 ± 5.07	2.88 ± 1.13	0.16 ± 0.12
	E10	6	22.64 ± 1.74	13.00 ± 6.10	2.50 ± 1.38	0.21 ± 0.21
(*b*) Geographic variation, *G. affinis*	C1	10	26.74 ± 2.12	15.50 ± 2.80	1.80 ± 0.63	0.16 ± 0.15
	C2	10	29.31 ± 2.01	39.30 ± 5.76	3.40 ± 1.35	0.31 ± 0.15
	C3	9	27.50 ± 4.96	34.00 ± 20.30	2,67 ± 1.32	0.16 ± 0.15
	C4	10	26.74 ± 1.13	14.30 ± 2.54	2.40 ± 0.84	0.17 ± 0.13
	C5	6	31.37 ± 3.99	46.50 ± 18.86	3.00 ± 1.10	0.29 ± 0.12
	C6	10	32.65 ± 0.94	54.40 ± 7.83	2.50 ± 1.18	0.18 ± 0.15
	C7	10	33.46 ± 3.18	31.10 ± 12.90	1.90 ± 1.20	0.14 ± 0.16
	C8	10	32.17 ± 1.02	42.30 ± 12.59	2.70 ± 1.42	0.26 ± 0.19
	C9	10	20.27 ± 1.31	7.90 ± 1.20	1.80 ± 1.23	0.09 ± 0.14
	C10	10	33.25 ± 3.16	52.90 ± 26.02	2.20 ± 0.79	0.19 ± 0.14
	C11	10	32.55 ± 2.99	22.40 ± 5.38	1.40 ± 0.70	0.05 ± 0.13
(*c*) Temporal variation, Ankang	May	10	24.78 ± 3.94	23.70 ± 5.70	2.90 ± 1.10	0.24 ± 0.15
	Jun.	10	29.94 ± 1.17	34.70 ± 4.60	3.00 ± 1.16	0.18 ± 0.12
	Jul.	10	25.15 ± 1.57	23.67 ± 6.38	1.44 ± 0.73	0.11 ± 0.18
	Aug.	10	27.18 ± 2.48	26.80 ± 5.77	2.20 ± 1.03	0.16 ± 0.16
	Sept.	10	27.22 ± 2.76	24.20 ± 6.83	2.60 ± 0.97	0.27 ± 0.16
	Oct.	10	27.19 ± 2.83	19.40 ± 3.10	1.70 ± 1.06	0.06 ± 0.11
(*d*) Temporal variation, Beihai	Apr.	10	25.03 ± 2.75	9.20 ± 1.81	2.50 ± 0.97	0.22 ± 0.13
	May	10	26.86 ± 1.82	10.70 ± 2.45	2.90 ± 0.99	0.19 ± 0.17
	Jun.	10	32.55 ± 2.99	22.40 ± 5.38	2.80 ± 0.79	0.27 ± 0.14
	Jul.	10	23.49 ± 2.56	8.80 ± 2.25	2.70 ± 1.06	0.20 ± 0.19
	Aug.	10	23.59 ± 2.14	9.80 ± 2.66	2.50 ± 0.53	0.17 ± 0.13
	Sept.	10	28.52 ± 3.88	18.90 ± 7.82	3.50 ± 1.27	0.28 ± 0.12

Table C.6. Information on primers (forward: above, reverse: below) used to amplify eight nuclear microsatellite loci.

Locus	**Primer 5' - 3'**	**Repeat unit**	**Size range [bp]**	**Dye group**	**References**
Gaaf22	ATGCGACCTGAAACTTCTGC CCGAGGTCCTTGAGGTTTATAG	ATC	233-275	FAM	Purcell et al. 2011
Gafµ2	CTCCAAACACACGTCCAATAATC AGTTTCCCCAGCCGTTCAT	CA	141-171	FAM	Spencer et al. 1999
Gafµ3	CTCAGCCGTCATTTAGTCTCAT GCACATAACATGGAAACAGTAAAC	GT	232-278	ROX	Spencer et al. 1999
Gafµ4	ACAACGGAGACCTGCTGGAGTGG CGCGAACCGTCCGTTATCCGTA	CT	212-278	TAMRA	Spencer et al. 1999
Gaaf7	TCCATCCCATTATGACCACAG GCACTTAGAAATGCCTCGC	AATC	131-299	ROX	Purcell et al. 2011
Gaaf10	GAACTGAACCACCCAAAGGC TCCATCTGGAGACAGGTGTG	ATCC	216-368	HEX	Purcell et al. 2011
Gaaf13	ACTTGGTGGCAGATTTCAGG AAGGAAACAACATGCTGGC	GATT	123-207	FAM	Purcell et al. 2011
Gafµ7	CACAGAACAACACAGAAACTGGAGG TGCCGATGGATGTTCCTGTTAG	AG	145-181	HEX	Spencer et al. 1999

Table C.7. Descriptive statistics (mean ± SD) of life-history traits in *G. affinis* from the (*a*) Ankang and (*b*) Beihai populations across the reproductive season of 2016. For abbreviations of life-history traits see main text.

	Sex	**Month**	***N***	**SL [mm]**	**Somatic lean weight [mg]**	**Fat content [%]**	**Fecundity**	**RA/ GSI [%]**	**Embryo lean weight [mg]**	**Embryo fat content [%]**
(*a*) Ankang	Female	May	16	28.97 ± 3.82	102.16 ± 43.32	4.62 ± 1.85	37.31 ± 17.02	20.31 ± 2.64	0.77 ± 0.11	1.96 ± 1.58
		Jun.	18	30.66 ± 1.61	117.05 ± 19.06	5.18 ± 2.07	32.72 ± 7.23	20.13 ± 3.57	0.89 ± 0.15	3.35 ± 2.48
		Jul.	24	32.10 ± 1.80	136.71 ± 24.26	5.91 ± 1.35	32.21 ± 8.45	18.90 ± 2.82	0.96 ± 0.12	3.98 ± 2.40
		Aug.	25	31.21 ± 1.97	115.13 ± 21.74	3.37 ± 2.13	25.56 ± 6.49	17.63 ± 3.14	0.94 ± 0.12	1.87 ± 1.56
		Sept.	21	23.82 ± 2.66	48.47 ± 16.66	1.77 ± 0.80	15.10 ± 7.50	18.76 ± 4.68	0.74 ± 0.10	1.50 ± 3.34
		Oct.	19	23.53 ± 2.66	48.41 ± 17.57	1.20 ± 0.62	9.68 ± 7.90	11.77 ± 4.81	0.72 ± 0.15	0.48 ± 0.44
	Male	May	30	20.23 ± 1.77	29.28 ± 9.39	1.70 ± 0.43	—	2.73 ± 0.47	—	—
		Jun.	30	21.32 ± 1.68	34.43 ± 8.85	2.93 ± 0.62	—	2.32 ± 0.34	—	—
		Jul.	30	20.61 ± 1.28	31.68 ± 6.24	2.28 ± 0.60	—	1.88 ± 0.43	—	—
		Aug.	30	20.56 ± 1.16	29.73 ± 5.13	4.95 ± 0.50	—	2.10 ± 0.37	—	—
		Sept.	30	19.29 ± 1.22	23.57 ± 4.43	4.25 ± 0.83	—	2.07 ± 0.31	—	—
		Oct.	17	20.47 ± 2.18	29.92 ± 10.25	4.32 ± 0.61	—	2.36 ± 0.54	—	—
(*b*) Beihai	Female	Apr.	25	26.90 ± 1.61	63.90 ± 13.04	3.60 ± 1.52	14.68 ± 5.67	20.30 ± 3.99	1.16 ± 0.16	4.08 ± 2.36
		May	27	26.88 ± 1.70	68.74 ± 12.55	1.45 ± 0.55	13.81 ± 5.32	14.55 ± 3.96	0.86 ± 0.15	3.30 ± 5.90
		Jun.	25	28.97 ± 1.70	62.07 ± 12.63	1.07 ± 0.74	18.84 ± 8.61	13.50 ± 3.89	0.76 ± 0.12	0.51 ± 0.62
		Jul.	22	24.18 ± 3.42	39.49 ± 16.75	1.50 ± 0.74	9.82 ± 2.20	15.06 ± 3.32	0.72 ± 0.18	0.50 ± 0.49
		Aug.	25	25.91 ± 2.72	49.85 ± 19.54	2.07 ± 0.99	17.60 ± 8.15	15.17 ± 5.89	0.73 ± 0.15	1.62 ± 0.94
		Sept.	21	27.82 ± 2.35	81.67 ± 21.45	4.56 ± 1.11	19.14 ± 5.21	17.17 ± 4.78	0.92 ± 0.17	3.04 ± 1.51
	Male	Apr.	19	20.14 ± 1.32	25.59 ± 5.06	2.42 ± 0.85	—	2.60 ± 0.48	—	—
		May	28	18.51 ± 1.47	18.58 ± 4.11	1.33 ± 0.42	—	1.63 ± 0.69	—	—
		Jun.	26	18.76 ± 1.97	20.48 ± 6.14	1.19 ± 0.71	—	2.02 ± 0.82	—	—
		Jul.	30	17.98 ± 1.73	16.43 ± 6.49	0.66 ± 0.66	—	1.85 ± 0.75	—	—
		Aug.	30	16.75 ± 1.17	13.09 ± 2.86	0.93 ± 0.48	—	1.74 ± 0.50	—	—
		Sept.	25	17.44 ± 0.87	18.16 ± 3.22	4.17 ± 1.52	—	1.97 ± 0.50	—	—

Table C.8. General linear models (GLM) on (*a*, *b*) population differences and (*c*, *d*) monthly differences in numbers of sires and reproductive skew of invasive mosquitofish. Statistically significant effects are shown in bold. Non-significant interaction terms that were removed from the final model are shown in brackets. Effect strengths were calculated as Wilk's partial η^2.

Dependent variable	**Source**	***d.f.***	***F***	***P***	**Partial η^2**
(*a*) Numbers of sires, geographic variation	**Species**	**1**	**12.946**	**< 0.001**	**0.074**
	Population(species)	**19**	**3.232**	**< 0.001**	**0.274**
	Fecundity	**1**	**3.974**	**0.048**	**0.024**
	Species × fecundity	**1**	**3.950**	**0.049**	**0.024**
	Error	163			
(*b*) Reproductive skew, geographic variation	Species	1	2.97	0.087	0.018
	Population(species)	19	1.429	0.120	0.142
	Fecundity	**1**	**5.668**	**0.018**	**0.033**
	[Species × fecundity]	1	< 0.001	0.995	< 0.001
	Error	164			
(*c*) Numbers of sires, temporal variation	**Population**	**1**	**6.960**	**0.010**	**0.061**
	Month(population)	10	1.632	0.107	0.132
	Fecundity	1	1.637	0.203	0.015
	[Population × fecundity]	1	2.587	0.111	0.024
	Error	107			
(*d*) Reproductive skew, temporal variation	**Population**	**1**	**6.339**	**0.013**	**0.056**
	Month(population)	10	1.554	0.131	0.127
	Fecundity	1	2.328	0.130	0.021
	[Population × fecundity]	1	0.785	0.378	0.007
	Error	107			

Table C.9. Information on microsatellite markers and occurrence of multiple paternity of invasive mosquitofish. Shown are the mean polymorphic information content (PIC), calculated using 8 nuclear microsatellite loci, combined exclusion probabilities (E-2P) when one parent (i.e., the mother) is known, average number of putative sires per brood (Number of sires), and frequencies of multiply sired broods [%] according to Colony (Wang 2004, Jones and Wang 2010). (*a*) Results for the ten populations included in our analysis of geographic variation in *G. holbrooki*. (*b*) Results for the eleven populations included in our analysis of geographic variation in *G. affinis*. (*c*) Results for monthly samples in Ankang and (*d*) Beihai.

	Population/ Month		**PIC**	**E-2P [%]**	**Number of sires**	**Multiply sired broods [%]**
(*a*) Geo. variation, *G. holbrooki*	E1	L. di Fimon C/W - IT	0.395	93.56	5.00 ± 1.41	100
	E2	L. di Fimon S - IT	0.382	90.00	4.63 ± 2.45	75
	E3	Porto Cesareo - IT	0.600	98.59	1.40 ± 0.55	40
	E4	Gualta - ES	0.497	95.67	2.63 ± 1.69	63
	E5	El Palmar - ES	0.467	95.43	3.30 ± 1.16	90
	E6	Río Xuquer - ES	0.516	96.42	2.11 ± 0.60	89
	E7	Río Vaca - ES	0.430	92.14	2.60 ± 1.27	80
	E8	Lebrija NE - ES	0.562	98.80	2.11 ± 1.05	56
	E9	Lebrija W - ES	0.502	98.10	2.88 ± 1.13	88
	E10	Doñana - ES	0.574	98.61	2.50 ± 1.38	67
(*b*) Geo. variation, *G. affinis*	C1	Baoding	0.588	98.66	2.50 ± 1.18	70
	C2	Ankang	0.602	99.27	3.40 ± 1.35	90
	C3	Shanghai	0.717	99.83	2.67 ± 1.32	67
	C4	Chengdu	0.700	99.81	2.40 ± 0.84	90
	C5	Wuhan	0.563	97.85	3.00 ± 1.10	100
	C6	Hangzhou	0.588	98.82	1.80 ± 0.63	80
	C7	Chongqing	0.566	98.60	1.90 ± 1.20	60
	C8	Kunming	0.689	99.74	2.70 ± 1.42	90
	C9	Shenzhen	0.662	99.57	1.80 ± 1.23	50
	C10	Hepu	0.542	97.79	2.20 ± 0.79	80
	C11	Beihai	0.477	96.58	1.40 ± 0.70	30
(*c*) Temp. variation, Ankang	5	May	0.519	96.89	2.90 ± 1.10	100
	6	June	0.675	96.87	3.00 ± 1.16	90
	7	July	0.634	99.38	1.44 ± 0.73	33
	8	August	0.638	99.38	2.20 ± 1.03	70
	9	September	0.407	92.68	2.60 ± 0.97	90
	10	October	0.658	99.38	1.70 ± 1.06	40
(*d*) Temp. variation, Beihai	4	April	0.293	79.06	2.50 ± 0.97	90
	5	May	0.301	81.74	2.90 ± 0.99	90
	6	June	0.309	82.51	2.80 ± 0.79	100
	7	July	0.282	76.47	2.70 ± 1.06	90
	8	August	0.311	83.11	2.50 ± 0.53	100
	9	September	0.323	81.62	3.50 ± 1.27	100

Table C.10. Genetic diversity of the ten *G. holbrooki* populations included in the analysis of geographic variation in multiple paternity. For each population and locus sample size (*N*), observed (H_O) and expected (H_E) heterozygosities, allelic richness (A_R) and deviation from Hardy-Weinberg equilibrium (*HWE*) are presented. *HWE* was calculated using GENEPOP v.4.2 (www.genepop.curtin.edu.au; Raymond and Rousset 1995, Rousset 2008). Values < 0.05, representing significant deviation from *HWE*, are highlighted in bold. Mean pop. indicates the mean value across populations, while mean loci indicates the mean value across loci.

Population		E1	E2	E3	E4	E5	E6	E7	E8	E8	E10	Mean pop
N		8	8	5	8	10	9	10	9	8	6	
H_O	Gaaf22	0.25	0.50	0.80	0.63	0.70	0.78	0.60	0.89	0.38	0.50	0.60
	Gafμ2	0.13	0.13	0.20	0.25	0.20	0.67	0.00	0.56	0.25	0.33	0.27
	Gafμ3	0.63	0.75	0.60	0.75	0.30	0.78	0.80	0.44	0.38	0.67	0.61
	Gafμ4	0.25	0.00	0.80	0.50	0.50	0.89	0.30	0.78	0.75	0.20	0.50
	Gaaf7	0.13	0.13	0.80	0.50	0.40	0.33	0.50	0.44	0.63	0.33	0.42
	Gaaf10	0.38	0.25	0.80	0.63	0.80	0.63	0.70	0.78	0.88	0.67	0.65
	Gaaf13	0.88	0.63	0.80	0.63	0.50	0.63	1.00	0.89	0.88	0.67	0.75
	Gafuμ7	0.50	0.50	0.80	0.50	0.50	0.88	0.70	0.22	0.75	0.50	0.59
	Mean loci	0.39	0.36	0.70	0.55	0.49	0.70	0.58	0.63	0.61	0.48	0.55
H_E	Gaaf22	0.22	0.41	0.70	0.66	0.57	0.59	0.59	0.68	0.49	0.60	0.55
	Gafμ2	0.12	0.12	0.50	0.22	0.18	0.44	0.00	0.44	0.23	0.28	0.25
	Gafμ3	0.53	0.55	0.62	0.51	0.26	0.75	0.62	0.51	0.32	0.69	0.54
	Gafμ4	0.41	0.22	0.64	0.51	0.72	0.60	0.48	0.82	0.77	0.62	0.58
	Gaaf7	0.12	0.12	0.66	0.62	0.34	0.43	0.54	0.44	0.60	0.54	0.44
	Gaaf10	0.43	0.40	0.64	0.65	0.79	0.69	0.53	0.71	0.68	0.57	0.61
	Gaaf13	0.85	0.73	0.76	0.70	0.71	0.67	0.69	0.78	0.86	0.76	0.75
	Gafμ7	0.55	0.55	0.64	0.50	0.41	0.57	0.54	0.44	0.47	0.67	0.53
	Mean loci	0.40	0.39	0.65	0.54	0.50	0.59	0.50	0.60	0.55	0.59	0.53
A_R	Gaaf22	1.77	2.53	3.78	3.50	2.79	2.82	2.65	3.77	2.00	3.49	2.91
	Gafμ2	1.50	1.50	2.00	1.77	1.65	1.99	1.00	2.74	2.00	1.91	1.81
	Gafμ3	2.73	2.50	2.98	2.49	1.81	3.80	2.80	3.00	2.27	4.00	2.84
	Gafμ4	2.53	1.77	3.60	2.49	3.83	2.89	2.86	5.48	4.70	2.98	3.31
	Gaaf7	1.50	1.50	3.76	2.89	2.21	2.38	2.40	1.99	2.77	2.67	2.41
	Gaaf10	1.99	2.40	2.98	3.36	4.35	3.75	3.01	3.93	3.93	2.89	3.26
	Gaaf13	5.90	4.43	5.18	3.99	3.69	3.96	3.59	4.89	5.87	5.24	4.67
	Gafμ7	2.50	3.17	3.60	3.27	2.61	2.75	2.40	2.74	2.00	3.98	2.90
	Mean loci	2.55	2.47	3.48	2.97	2.87	3.04	2.59	3.57	3.19	3.39	3.01
HWE	Gaaf22	1.00	1.00	0.69	0.72	1.00	1.00	1.00	0.10	0.53	0.16	0.72
	Gafμ2	**0.00**	**0.00**	0.36	1.00	1.00	0.46	**0.00**	1.00	1.00	1.00	0.58
	Gafμ3	1.00	0.48	0.62	0.63	1.00	0.83	0.65	0.49	1.00	1.00	0.77
	Gafμ4	0.13	0.07	1.00	1.00	**0.01**	0.20	0.10	0.65	0.48	0.05	0.37
	Gaaf7	**0.00**	**0.00**	0.74	0.37	1.00	0.54	1.00	1.00	0.54	0.15	0.54
	Gaaf10	1.00	0.37	0.49	0.36	0.66	0.49	0.78	0.59	0.82	1.00	0.66
	Gaaf13	0.73	0.38	0.80	0.28	0.13	0.37	0.09	0.38	0.28	0.16	0.36
	Gafμ7	1.00	0.16	1.00	0.56	1.00	0.33	0.73	**0.04**	0.44	0.26	0.55
	Mean loci	0.61	0.31	0.71	0.62	0.72	0.53	0.54	0.53	0.64	0.47	0.57

Table C.11. Genetic diversity of the eleven *G. affinis* populations included in the analysis of geographic variation in multiple paternity. For each population and locus sample size (*N*), observed (H_O) and expected (H_E) heterozygosities, allelic richness (A_R) and deviation from Hardy-Weinberg equilibrium (*HWE*) are presented. *HWE* was calculated using GENEPOP v.4.2. Values < 0.05, i.e., significant deviations from *HWE*, are highlighted in bold. Mean pop. indicates the mean value across populations, while Mean loci indicates the mean value across loci.

Population		**C1**	**C2**	**C3**	**C4**	**C5**	**C6**	**C7**	**C8**	**C9**	**C10**	**C11**	**Mean pop.**
N		10	10	9	10	6	10	10	10	10	10	10	
H_O	Gaaf22	0.70	0.70	0.78	0.50	0.83	0.50	0.60	0.60	0.60	0.90	0.50	0.66
	Gafµ2	0.80	0.80	0.89	0.40	0.83	0.20	0.70	0.80	0.50	0.30	0.40	0.60
	Gafµ3	0.70	0.80	1.00	0.10	0.83	0.60	0.40	1.00	0.50	0.80	0.10	0.62
	Gafµ4	0.70	0.90	0.89	0.10	0.83	0.50	0.60	0.50	0.60	0.90	0.10	0.60
	Gaaf7	0.70	1.00	0.89	0.20	0.83	0.80	0.40	1.00	0.70	0.90	0.20	0.69
	Gaaf10	0.50	1.00	0.89	0.40	0.67	0.40	0.30	1.00	0.70	0.90	0.40	0.65
	Gaaf13	0.90	0.80	0.78	0.60	1.00	0.50	0.70	1.00	0.90	1.00	0.60	0.80
	Gafµ7	0.80	0.80	0.44	0.90	0.50	0.70	0.70	0.80	0.60	0.90	0.90	0.73
	Mean loci	0.73	0.85	0.82	0.40	0.79	0.53	0.55	0.84	0.64	0.83	0.40	0.67
H_E	Gaaf22	0.58	0.62	0.76	0.67	0.54	0.66	0.59	0.72	0.69	0.61	0.67	0.64
	Gafµ2	0.69	0.62	0.72	0.54	0.58	0.41	0.69	0.74	0.60	0.27	0.54	0.58
	Gafµ3	0.62	0.79	0.80	0.10	0.69	0.61	0.56	0.84	0.70	0.72	0.10	0.59
	Gafµ4	0.79	0.89	0.76	0.59	0.57	0.82	0.79	0.60	0.88	0.69	0.59	0.72
	Gaaf7	0.69	0.73	0.77	0.18	0.54	0.76	0.54	0.82	0.77	0.65	0.18	0.60
	Gaaf10	0.40	0.72	0.80	0.59	0.60	0.34	0.40	0.58	0.67	0.72	0.59	0.58
	Gaaf13	0.77	0.59	0.70	0.76	0.76	0.66	0.84	0.81	0.78	0.69	0.76	0.74
	Gafµ7	0.56	0.59	0.54	0.68	0.42	0.67	0.58	0.69	0.59	0.54	0.68	0.59
	Mean loci	0.64	0.69	0.73	0.51	0.59	0.61	0.62	0.72	0.71	0.61	0.51	0.63
A_R	Gaaf22	3.00	4.00	5.00	7.44	6.00	4.00	4.29	4.61	4.00	4.00	4.00	4.58
	Gafµ2	4.00	5.00	5.00	7.44	5.00	6.73	4.00	5.00	4.00	3.60	3.99	4.89
	Gafµ3	5.44	7.21	11.10	6.58	6.00	3.98	3.00	8.61	6.00	6.81	2.00	6.07
	Gafµ4	9.44	13.23	11.32	13.11	4.00	9.54	6.00	10.20	11.74	14.29	6.58	9.95
	Gaaf7	5.00	11.57	10.98	5.84	5.00	7.94	3.00	9.65	5.00	4.00	3.97	6.54
	Gaaf10	3.00	9.16	8.57	8.75	7.00	5.71	3.65	8.26	5.92	6.99	5.00	6.55
	Gaaf13	6.99	9.25	10.31	10.56	7.00	6.91	7.00	10.41	6.98	9.51	8.96	8.54
	Gafµ7	3.00	3.62	5.90	8.77	5.00	6.00	3.00	5.61	4.00	5.52	4.98	5.03
	Mean loci	4.98	7.88	8.52	8.56	5.63	6.35	4.24	7.79	5.95	6.84	4.93	6.52
HWE	Gaaf22	1.00	1.00	0.28	0.09	0.64	0.39	1.00	0.32	**0.02**	0.35	0.09	0.47
	Gafµ2	0.73	0.90	0.95	0.05	1.00	0.05	0.62	0.53	0.49	1.00	0.06	0.58
	Gafµ3	0.75	0.99	1.00	**0.00**	1.00	0.16	0.24	0.96	0.07	0.39	**0.00**	0.51
	Gafµ4	0.41	0.72	0.82	**0.00**	0.39	**0.00**	**0.03**	0.18	**0.00**	0.48	**0.00**	0.28
	Gaaf7	0.34	0.91	0.96	1.00	0.64	0.32	0.26	0.33	0.50	0.75	1.00	0.64
	Gaaf10	1.00	0.10	0.81	0.07	0.51	1.00	0.47	**0.01**	0.88	0.07	0.07	0.45
	Gaaf13	0.50	1.00	0.65	0.06	0.53	0.07	0.44	0.33	0.42	**0.02**	0.05	0.37
	Gafµ7	0.34	0.77	0.52	0.78	1.00	0.09	1.00	0.74	1.00	0.05	0.79	0.64
	Mean loci	0.63	0.80	0.75	0.26	0.71	0.26	0.51	0.42	0.42	0.39	0.26	0.49

Table C.12. Genetic diversity *G. affinis* from Ankang used for the analysis of temporal variation in multiple paternity. For each month and locus sample size (*N*), observed (H_O) and expected (H_E) heterozygosities, allelic richness (A_R) and Hardy-Weinberg equilibrium (*HWE*) are presented. *HWE* was calculated using GENEPOP v.4.2. Values lower than 0.05, which represent significant deviation from HWE, are highlighted in bold. Mean months indicates the mean value across monthly samplings, while Mean loci indicates the mean value across loci.

Ankang		**May**	**June**	**July**	**August**	**September**	**October**	**Mean months**
N		10	10	10	10	10	10	
H_O	Gaaf22	0.46	0.66	0.50	0.56	0.67	0.63	0.58
	Gafμ2	0.42	0.63	0.61	0.65	0.29	0.67	0.54
	Gafμ3	0.24	0.59	0.49	0.64	0.08	0.55	0.43
	Gafμ4	0.34	0.68	0.59	0.66	0.54	0.57	0.56
	Gaaf7	0.41	0.51	0.62	0.48	0.14	0.48	0.44
	Gaaf10	0.64	0.31	0.78	0.66	0.58	0.49	0.58
	Gaaf13	0.46	0.14	0.36	0.49	0.31	0.52	0.38
	Gafμ7	0.53	0.70	0.61	0.57	0.71	0.34	0.58
	Mean loci	0.44	0.53	0.57	0.59	0.41	0.53	0.51
H_E	Gaaf22	0.72	0.67	0.63	0.63	0.65	0.68	0.66
	Gafμ2	0.42	0.64	0.59	0.64	0.29	0.72	0.55
	Gafμ3	0.31	0.79	0.57	0.82	0.09	0.75	0.55
	Gafμ4	0.56	0.78	0.83	0.79	0.53	0.77	0.71
	Gaaf7	0.49	0.66	0.67	0.72	0.13	0.69	0.56
	Gaaf10	0.61	0.77	0.81	0.79	0.66	0.75	0.73
	Gaaf13	0.72	0.13	0.54	0.49	0.47	0.65	0.50
	Gafμ7	0.66	0.70	0.74	0.62	0.71	0.63	0.68
	Mean loci	0.56	0.64	0.67	0.69	0.44	0.70	0.62
A_R	Gaaf22	4.00	7.00	4.00	4.00	4.00	4.00	4.50
	Gafμ2	4.00	7.00	4.00	4.00	4.00	5.00	4.67
	Gafμ3	3.00	11.00	5.00	8.00	4.00	5.00	6.00
	Gafμ4	5.00	8.00	8.00	6.00	6.00	7.00	6.67
	Gaaf7	6.00	8.00	8.00	5.00	3.00	7.00	6.17
	Gaaf10	6.00	10.00	10.00	6.00	7.00	6.00	7.50
	Gaaf13	9.00	4.00	6.00	6.00	6.00	8.00	6.50
	Gafμ7	5.00	5.00	5.00	4.00	5.00	5.00	4.83
	Mean loci	5.25	7.50	6.25	5.38	4.88	5.88	5.85
HWE	Gaaf22	0.06	1.00	0.86	1.00	0.36	1.00	0.71
	Gafμ2	1.00	0.90	1.00	0.67	**0.00**	0.44	0.67
	Gafμ3	0.05	0.99	**0.01**	**0.00**	**0.00**	0.96	0.33
	Gafμ4	0.05	0.69	0.18	0.06	1.00	0.10	0.35
	Gaaf7	0.12	0.91	0.32	0.41	0.48	0.11	0.39
	Gaaf10	1.00	0.10	0.26	0.91	0.22	**0.02**	0.42
	Gaaf13	0.14	1.00	0.16	**0.03**	**0.00**	0.11	0.24
	Gafμ7	0.19	0.77	0.74	**0.01**	0.19	0.60	0.42
	Mean loci	0.33	0.79	0.44	0.39	0.28	0.42	0.44

Table C.13. Genetic diversity of *G. affinis* from Beihai used for the analysis of temporal variation in multiple paternity. For each month and locus sample size (*N*), observed (H_O) and expected (H_E) heterozygosities, allelic richness (A_R) and deviation from Hardy-Weinberg equilibrium (*HWE*) are presented. *HWE* was calculated using GENEPOP v.4.2. Values < 0.05 indicate significant deviation from *HWE* and are highlighted in bold. Mean months indicates the mean value across monthly samplings, while Mean loci indicates the mean value across loci.

Beihai		**April**	**May**	**June**	**July**	**August**	**September**	**Mean months**
N		10	10	10	10	10	10	
H_O	Gaaf22	0.59	0.69	0.58	0.60	0.64	0.72	0.64
	Gafµ2	0.34	0.32	0.36	0.38	0.31	0.52	0.37
	Gafµ3	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Gafµ4	0.50	0.49	0.35	0.26	0.34	0.66	0.43
	Gaaf7	0.13	0.00	0.05	0.08	0.08	0.15	0.08
	Gaaf10	0.23	0.48	0.48	0.55	0.45	0.37	0.43
	Gaaf13	0.63	0.77	0.78	0.67	0.75	0.69	0.72
	Gafµ7	0.35	0.30	0.33	0.32	0.49	0.51	0.38
	Mean loci	0.35	0.38	0.37	0.36	0.38	0.45	0.38
H_E	Gaaf22	0.60	0.59	0.49	0.58	0.61	0.57	0.57
	Gafµ2	0.32	0.30	0.37	0.32	0.29	0.39	0.33
	Gafµ3	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Gafµ4	0.45	0.43	0.40	0.23	0.28	0.52	0.39
	Gaaf7	0.12	0.00	0.05	0.08	0.08	0.13	0.08
	Gaaf10	0.20	0.43	0.42	0.46	0.42	0.38	0.39
	Gaaf13	0.66	0.69	0.71	0.53	0.71	0.58	0.65
	Gafµ7	0.33	0.27	0.35	0.38	0.40	0.41	0.36
	Mean loci	0.33	0.34	0.35	0.32	0.35	0.37	0.34
A_R	Gaaf22	3.00	4.00	3.00	4.00	3.00	3.00	3.33
	Gafµ2	3.00	3.00	2.00	2.00	2.00	2.00	2.33
	Gafµ3	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	Gafµ4	3.00	3.00	3.00	4.00	2.00	3.00	3.00
	Gaaf7	2.00	1.00	2.00	2.00	2.00	2.00	1.83
	Gaaf10	3.00	4.00	4.00	4.00	3.00	3.89	3.65
	Gaaf13	4.00	4.00	5.89	4.00	6.00	5.00	4.82
	Gafµ7	3.00	2.00	3.00	3.00	3.99	3.00	3.00
	Mean loci	2.75	2.75	2.99	3.00	2.87	2.86	2.87
HWE	Gaaf22	1.00	0.76	0.33	1.00	0.64	0.36	0.68
	Gafµ2	1.00	1.00	1.00	0.16	1.00	1.00	0.86
	Gafµ3	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Gafµ4	0.68	1.00	1.00	1.00	1.00	0.68	0.89
	Gaaf7	0.00	0.00	0.00	1.00	1.00	0.00	0.33
	Gaaf10	0.00	1.00	1.00	1.00	1.00	1.00	0.83
	Gaaf13	0.76	0.89	0.55	1.00	0.08	1.00	0.71
	Gafµ7	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	Mean loci	0.56	0.71	0.61	0.77	0.71	0.63	0.66

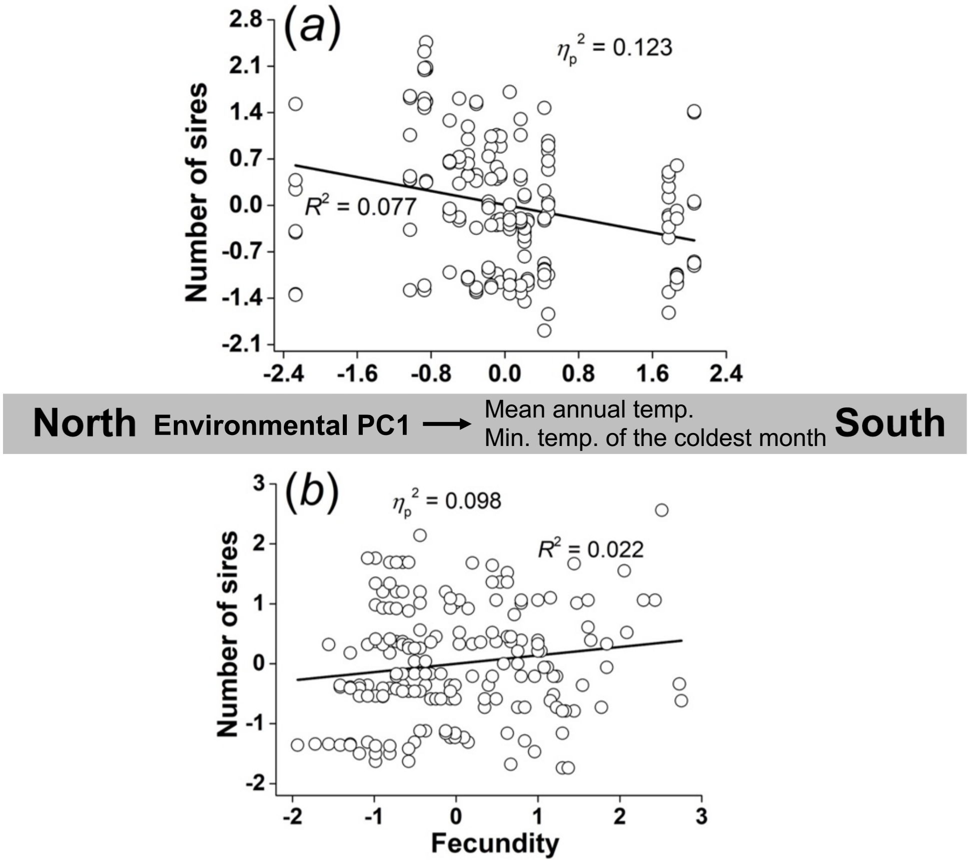

Figure C.1. Partial regression plots showing the effects of (*a*) climate and (*b*) fecundity on geographical variation in numbers of sires of both species of invasive mosquitofish (*G. holbrooki* and *G. affinis*). Dependent variables in panels (*a*) and (*b*) are residuals corrected for ‘fecundity’ and ‘environmental PC1’, respectively.

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Appendix D: Supplementary material for Chapter 5 – Phenotypic responses to natural and anthropogenic oil pollution in Trinidadian guppies (*Poecilia reticulata*).

Table D.1. Descriptive statistics (mean ± SE) of male *Poecilia reticulata* life-history traits.

Habitat type	Population	*N*	SL [mm]	Lean weight[a] [g]	Fat content[a] [%]	GSI [%]
Polluted	1	21	17.0 ± 0.2	0.018 ± 0.001	2.67 ± 0.87	3.17 ± 0.23
	4	14	14.9 ± 0.3	0.018 ± 0.001	1.11 ± 1.07	2.53 ± 0.12
	5	24	16.9 ± 0.3	0.019 ± 0.001	2.80 ± 0.82	2.65 ± 0.11
	6	15	17.4 ± 0.3	0.021 ± 0.001	0.37 ± 1.05	2.49 ± 0.14
	8	15	17.0 ± 0.3	0.023 ± 0.002	0.55 ± 1.03	2.19 ± 0.15
	10	18	15.0 ± 0.3	0.018 ± 0.001	4.66 ± 0.95	2.50 ± 0.20
	Total/Avg.	107	16.4 ± 0.2	0.019 ± 0.001	2.03 ± 0.39	2.62 ± 0.07
Non-polluted	2	24	16.0 ± 0.2	0.019 ± 0.001	4.73 ± 0.80	3.66 ± 0.20
	3	26	15.9 ± 0.2	0.018 ± 0.001	3.26 ± 0.77	3.35 ± 0.20
	7	15	14.9 ± 0.2	0.018 ± 0.001	3.33 ± 1.04	2.80 ± 0.12
	9	15	13.9 ± 0.7	0.015 ± 0.001	5.23 ± 1.11	2.22 ± 0.17
	11	14	17.3 ± 0.2	0.022 ± 0.001	0.46 ± 0.95	2.15 ± 0.13
	Total/Avg.	94	15.6 ± 0.2	0.018 ± 0.001	3.38 ± 0.43	2.98 ± 0.10

[a]: estimated marginal means for SL = 16.1 mm.

Table D.2. Descriptive statistics (mean ± SE) of female and offspring *Poecilia reticulata* life-history traits.

Habitat type	Population	*N*	SL [mm]	Lean weight[a] [g]	Fat content [%]	RA [%]	Fecundity[a]	Embryo lean weight [mg]	Embryo fat content [%]
Polluted	1	15	21.4 ± 0.5	0.045 ± 0.002	4.15 ± 0.62	13.82 ± 1.12	10.47 ± 1.13	0.65 ± 0.04	7.91 ± 1.39
	4	15	22.1 ± 0.3	0.053 ± 0.002	2.34 ± 0.44	14.72 ± 1.15	16.33 ± 1.12	0.54 ± 0.03	2.93 ± 0.55
	5	14	24.5 ± 0.6	0.063 ± 0.002	5.03 ± 1.26	9.38 ± 0.77	7.92 ± 1.17	0.77 ± 0.05	6.22 ± 1.42
	6	11	25.3 ± 0.6	0.062 ± 0.003	4.27 ± 0.62	12.44 ± 1.12	9.02 ± 1.39	0.95 ± 0.05	2.50 ± 0.63
	8	13	25.4 ± 0.4	0.068 ± 0.002	2.60 ± 0.41	9.56 ± 0.99	8.39 ± 1.30	0.83 ± 0.06	1.28 ± 0.46
	10	15	22.9 ± 0.4	0.065 ± 0.002	0.62 ± 0.26	13.92 ± 0.94	14.34 ± 1.12	0.74 ± 0.04	1.00 ± 0.38
	Total/Avg.	83	23.5 ± 0.2	0.059 ± 0.001	3.11 ± 0.32	12.40 ± 0.47	11.08 ± 0.50	0.73 ± 0.02	4.45 ± 0.68
Non-polluted	2	14	25.1 ± 0.6	0.061 ± 0.002	4.57 ± 1.12	17.84 ± 1.18	23.79 ± 1.25	0.63 ± 0.03	3.93 ± 1.35
	3	15	21.1 ± 0.5	0.055 ± 0.002	4.65 ± 0.84	9.86 ± 0.85	8.52 ± 1.15	0.70 ± 0.03	9.20 ± 1.71
	7	15	21.6 ± 0.5	0.057 ± 0.002	3.82 ± 0.66	12.22 ± 1.30	11.81 ± 1.13	0.68 ± 0.05	6.55 ± 1.65
	9	13	18.3 ± 0.6	0.058 ± 0.003	2.23 ± 0.68	9.42 ± 0.75	12.04 ± 1.44	0.47 ± 0.02	1.83 ± 0.42
	11	14	22.9 ± 0.4	0.061 ± 0.002	1.60 ± 0.42	7.18 ± 0.52	5.46 ± 1.15	0.82 ± 0.03	2.66 ± 0.65
	Total/Avg.	71	21.8 ± 0.4	0.058 ± 0.001	3.42 ± 0.37	11.32 ± 0.61	12.32 ± 0.54	0.67 ± 0.02	5.19 ± 0.70

[a]: estimated marginal means for SL = 22.7 mm, and embryonic stage of development = 25.9.

Table D.3. Overview of biotic and abiotic environmental parameters measured for guppy sampling sites.

Habitat type	Population	Latitude [N]	Longitude [W]	Water temperature [°C]	Dissolved oxygen [mg/L]	Salinity [ppt]	pH	Other species observed
Polluted	1	10.235	-61.625	27.7	3.29	0.83	6.69	*Anablepsoides hartii, Polycentrus schomburgkii*, *Rhyncops niger*, *Sternula superciliaris*
	4	10.199	-61.630	29.7	2.71	0.00	6.56	*A. hartii*
	5	10.196	-61.633	27.8	2.09	1.30	7.07	Freshwater shrimps (*Macrobrachium* sp.)
	6	10.190	-61.569	26.8	1.04	3.81	7.76	*A. hartii*, *Macrobrachium* sp.
	8	10.170	-61.567	26.1	0.73	3.42	7.77	*A. hartii*
	10	10.171	-61.681	29.7	1.00	5.05	7.47	*A. hartii*
Non-polluted	2	10.237	-61.614	32.2	3.20	0.11	6.97	Odonata nymphs
	3	10.202	-61.633	26.5	1.22	0.20	6.64	Odonata nymphs
	7	10.199	-61.597	28.7	1.42	0.56	7.46	*A. hartii*, *A. bimaculatus*, *Ardea herodias*
	9	10.173	-61.681	29.7	1.60	0.19	7.73	*A. hartii*, *A. bimaculatus*, *Hypostomus plecostomus*, *P. schomburgkii*, *Ardea herodias*, Odonata nymphs
	11	10.689	-61.290	26.7	6.90	0.15	8.01	*A. bimaculatus*, *H. plecostomus*, *Ardea herodias*, Odonata nymphs

Table D.4. Relative Warps (RWs) used in the body-shape analysis. Reported are Eigenvalues and % of variance explained by each RW.

RW	Eigenvalue	% Variance explained	Cumulative % var. explained
RW1	1.328	79.21	79.21
RW2	0.297	3.98	83.19
RW3	0.262	3.08	86.27
RW4	0.246	2.72	88.98
RW5	0.209	1.97	90.95

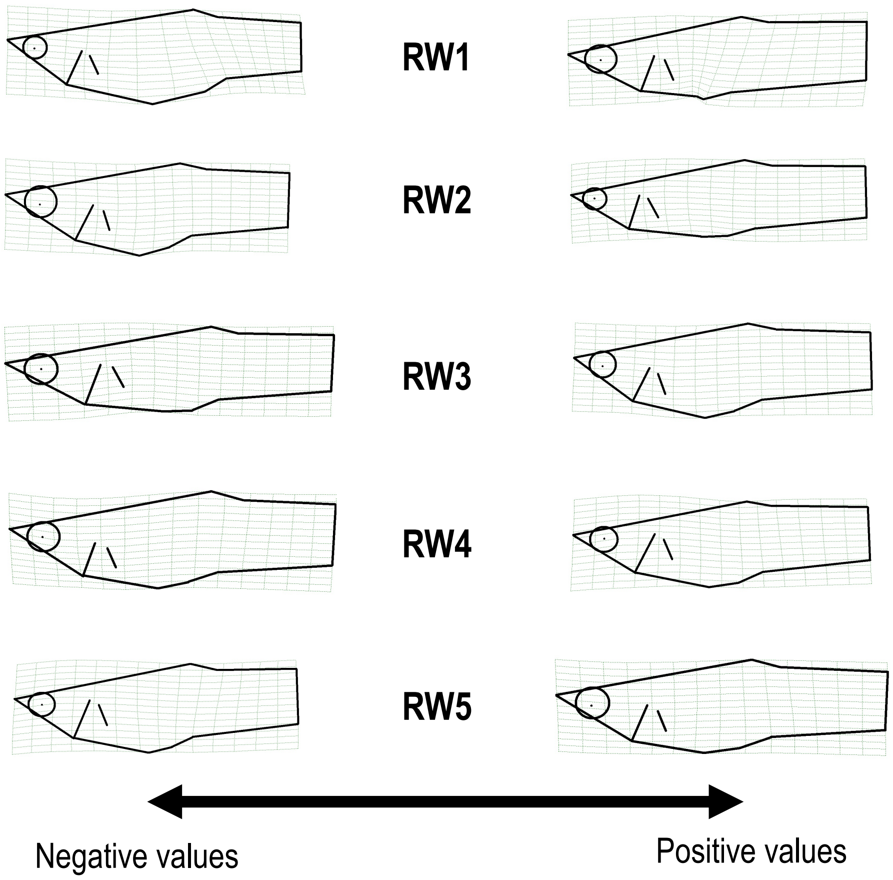

Figure D.1. Thin-plate spline representation of the 5 Relative Warps (RWs) used in the analysis of guppy body shape.

Appendix E: Life histories of guppies (*Poecilia reticulata* Peters, 1869; Poeciliidae) from the Pitch Lake in Trinidad

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Abstract

Trinidadian guppies (*Poecilia reticulata*) are able to adapt to various environmental conditions and are even among the few species that can tolerate extensive pollution. In the Pitch Lake of Trinidad they live in highly toxic waters due to natural seepage of oil and bitumen. In this paper, we describe phenotypic divergence in several life-history traits between guppies from the Pitch Lake and from a nearby reference site with waters not polluted by bitumen/oil. We show that guppies from the Pitch Lake were (i) smaller and (ii) had a higher reproductive investment than those from the reference site. Furthermore, they (iii) produced more and smaller offspring. These results are congruent with a scenario of high mortality caused probably by a combination of water toxicity and higher predation than at the reference site. We therefore propose the Pitch Lake as an ideal system to study the effects of long-term (natural) water pollution on fishes, which might provide interesting insights into adaptation to extreme environments, and might further help to predict fish responses to anthropogenic pollution.

Key-words

Life-history; Oil pollution; Predation; Phenotypic evolution.

Introduction

Among extremophile vertebrates (from the Latin term *extremus* and the Greek *philia*; roughly translating into “loving the extreme”), teleost fishes are particularly numerously represented and inhabit environments that are, for example, extreme due to salinities far above concentrations found in sea water, low oxygen content, low temperatures, the presence of toxins, or the absence of light (e.g., Gerday and Glansdorff 2009, Riesch et al. 2015b). While many of these habitats are naturally extreme to teleost fishes and can only be inhabited by highly specialized forms, more and more habitats, terrestrial and aquatic, are currently becoming extreme due to the impact of human activities on ecosystems in general and human-induced pollution in particular (e.g., Oziolor and Matson 2015, Reid et al. 2016, Hamilton et al. 2017).

The Pitch Lake (Fig. E.1) in South-West Trinidad is the largest and best-known asphalt lake in the world (Trinidad and Tobago National Commission for UNESCO 2011, Schelkle et al. 2012). It was created and is maintained by natural upwelling of oils and bitumen that contain a plethora of hydrocarbons, sulphur compounds and metals, as well as volcanic ash, at concentrations high enough to create an extremely toxic environment (Richardson 1912, Ponnamperuna and Pering 1967, World Health Organization Concise International Chemical Assessment Document 2005). Due to the impermeability of the bitumen, several interconnected permanent pools fed by rainwater are interspersed throughout the Pitch Lake. Here, a surprising number of plants and animals seem to thrive, despite the fact that previous studies also reported relatively high water temperatures exceeding

30°C, coupled with high acidity and salinity (e.g., Mohammed et al. 2010, Schelkle et al. 2012). Nonetheless, the organisms inhabiting the Pitch Lake pools include bacteria (Meckenstock et al. 2014), invertebrates (Schelkle et al. 2012), amphibians (Schelkle et al. 2012) and fishes like Hart's killifish *Rivulus hartii* Boulenger, 1890, Guyana leaffish, *Polycentrus schomburgkii* Müller & Troschel, 1849, and the guppy, *Poecilia reticulata* Peters, 1869 (Mohammed et al. 2010, Schelkle et al. 2012).

The Trinidadian guppy has been a model organism for evolutionary and behavioural ecology for decades, largely due to the fact that as a result of human introductions, guppies now have an almost global distribution, but are also easy to keep and maintain in the laboratory (Evans et al. 2011, Magurran 2005). Furthermore, guppies are among those fish that can tolerate extensive natural and human-induced pollution and toxicity (Araujo et al. 2009, Riesch et al. 2015b). The guppies at the Pitch Lake in particular have recently been used by Schelkle et al. (2012) to study the influence of the pitch-induced toxicity on parasite dynamics. The study showed that the Pitch Lake water effectively protects guppies from microbial and gyrodactylid parasite infections (see also the enemy release hypothesis; Williamson 1996). Also, since fishes seem to naturally produce enzymes that, to a certain extent, allow them to detoxify different hydrocarbons when exposed during pollution events (Lee et al. 1972, Neff et al. 1976, Ownby et al. 2002), there should be a high potential for fishes to colonize extreme, oil-polluted environments and even locally adapt to oil contamination. Such local adaptations of fishes to polluted waters has been described for several other species and localities (Reid et al. 2016, Hamilton et al. 2017).

In the current study, we analyze phenotypic divergence in life-history traits in Pitch Lake guppies by comparing them to a nearby reference population living in a roadside ditch outside of the oil- and bitumen-contaminated area of the Pitch Lake (Fig. E.1). A recent study by Rolshausen et al. (2015) revealed that oil-polluted waters negatively impact guppy survival. Moreover, we would further expect detoxification of hydrocarbons and other toxins associated with oil pollution to be energetically costly (e.g., Marchand et al. 2004, Passow et al. 2015). As a result, we expect guppies from the Pitch Lake to suffer increased mortality across age classes when compared to those from the reference population, and therefore express an *r*-selected phenotype, which should be associated with a decreased investment into growth and an increased investment into reproduction (Pianka 1970). In other words, we predict guppies from the Pitch Lake to be smaller (reduced standard length and size-specific lean weight) and to have less body fat than their counterparts from our non-polluted reference site, but to have a higher gonadosomatic index (GSI), and reproductive allocation (RA), and to produce more and smaller offspring (Reznick et al. 2002a). Alternatively, if larger offspring size were to convey a fitness advantage in oil polluted sites (as has been suggested for other environmental toxins such as hydrogen sulphide; Riesch et al. 2016) then Pitch Lake guppies should be characterized by larger offspring size coupled with a lower fecundity compared to guppies from non-polluted sites.

Materials and methods

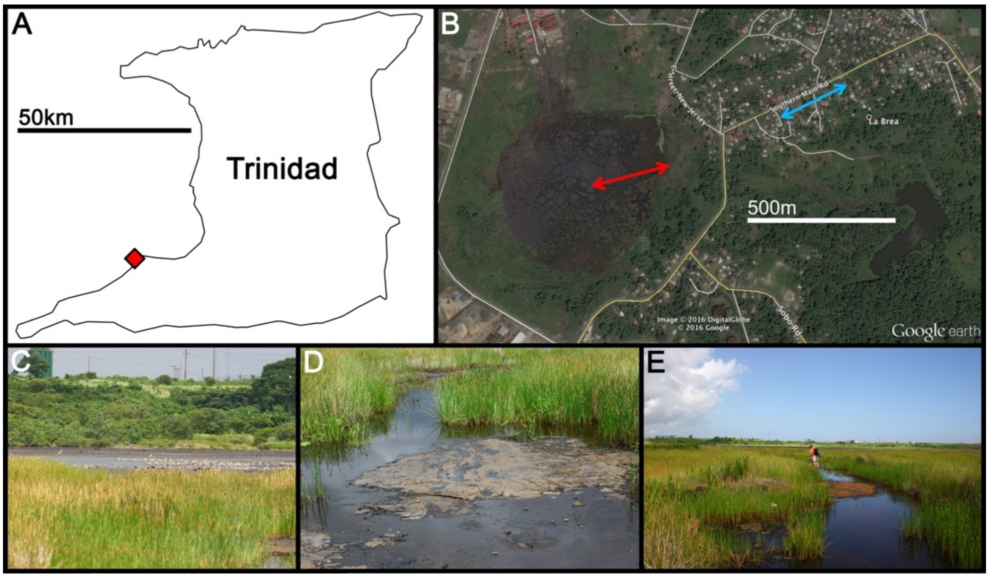

Figure E.1. (A) Location of our sample sites in Trinidad (red diamond); (B) Detailed view of collection transects. The red arrow indicates the general transect sampled within the Pitch Lake, while the blue arrow indicates the general transect sampled along a road-side ditch (our reference site); (C) large flocks of yellow-billed tern, *Sternula superciliaris*, and black skimmer, *Rynchops niger*, are resting and foraging on the Pitch Lake (i.e., the white 'dots' on the Pitch Lake); (D & E) Pitch Lake; (A) was created in R (R Core Team 2015), (B) was created using GOOGLE EARTH VS. 7.1.5.1557 (©2015 Google Inc., Mountain View, CA, USA), photos (C-E) by D. Bierbach.

We collected fish in June 2012 using small dip nets along two sampling transects in the Pitch Lake and the nearby reference site (Fig. E.1). Collections were made under a permit to M.S. kindly supplied by the Directorate of Fisheries of the Ministry of Food Production, Land and Marine Affairs of the Republic of Trinidad and Tobago, issued May 31st, 2012. Caught fish (for sample sizes and some habitat parameters, please refer to Table E.1) were immediately euthanized with an overdose of clove oil and preserved in 10% formalin solution. Similar to previous studies (e.g., Mohammed et al. 2010, Schelkle et al. 2012), we also found the Pitch Lake

Table E.1. Mean±SE of male and female life-history traits in wild-caught guppies from the Pitch Lake (pH: 6.06; water temperature: 29.2-33.9ºC; conductivity: 109µS) and a reference site (pH: 9.35; water temperature: 30.7ºC; conductivity: 499µS) in Trinidad. M: males; F: females; GSI: gonadosomatic index; RA: reproductive allocation; MI: matrotrophy index.

Population	Coordinates	Sex	N[a]	SL [mm]	Lean weight [mg][b]	Fat content [%]	Fecundity[b]	Estimated embryo dry weight at birth [mg][c]	Embryo fat content [%]	GSI [%]	RA[b] [%]	MI
Pitch Lake	N 10°14.084'	M	8 / 8	14.96 ± 0.17	19.90 ± 0.68	2.52 ± 1.26	-	-	-	2.74 ± 0.20	-	-
	W 61° 37.469'	F	11 / 18	20.84 ± 0.57	74.12 ± 2.80	4.24 ± 1.07	16.70 ± 2.54	0.49	20.62 ± 2.40	-	17.86 ± 1.91	0.53
Reference site	N 10° 14.262'	M	21 / 21	16.48 ± 0.22	21.66 ± 0.38	6.56 ± 0.80	-	-	-	1.96 ± 0.13	-	-
	W 61° 37.190'	F	24 / 25	24.93 ± 0.75	76.49 ± 1.79	12.80 ± 3.46	10.60 ± 1.62	0.92	19.97 ± 0.78	-	12.00 ± 1.28	0.78

[a] the numerator corresponds to reproductively active males and females & the denominator equals the total number of collected and dissected males and females.

[b] lean weight and fecundity are given as estimated marginal means corrected for differences in SL (evaluated at SL = 16.06 mm for males and SL = 23.65 mm for females), while RA-values are estimated marginal means corrected for differences in embryonic stage of development (evaluated at stage 25; Riesch et al. 2011).

[c] estimated embryo dry weight at birth is calculated using the slope and intercept from the regression between log-transformed embryonic dry mass and stage of development

to be highly acidic compared to our reference site, while water temperatures tended to be slightly higher in the Pitch Lake as well; although it was high (> 30ºC) also at our reference site (Table E.1). However, due to logistic constraints, we were not able to directly test for toxicity levels in each habitat. We therefore cannot completely rule out that even the reference site, due to its proximity to a main road, might suffer from some toxicity as a result of oil- and gasoline-runoff from the road.

Dissections to collect male, female, and offspring-related life-history traits followed well-established protocols (Reznick and Endler 1982, Riesch et al. 2013, 2016). We collected the following male and female life-history traits: standard length (SL [mm]), dry weight [mg], lean weight [mg], and fat content [%]. For males we also calculated the gonadosomatic index, GSI [%] (i.e., testis dry weight divided by the sum of reproductive tissue dry weight and somatic dry weight), while for females we quantified fecundity (number of developing offspring), offspring dry weight [mg], offspring lean weight [mg], offspring fat content [%], and reproductive allocation, RA [%] (i.e., offspring dry weight divided by the sum of offspring dry weight plus somatic dry weight).

Prior to statistical analyses, we log10-transformed (male and female SL, male and female lean weight, and embryo dry and lean weight), square root-transformed (fecundity), or arcsine(square root)-transformed (male and female fat content, male and female GSI, embryo fat content) all life-history variables, and conducted subsequent *z*-transformation to meet assumptions of statistical analyses (i.e., these transformations facilitated normality of model residuals). *Z*-transformed variables were used in all subsequent analyses unless specified otherwise.

We first tested for differences in body size (SL) by running an ANOVA with sex (male vs. female) and population (the Pitch Lake vs. reference site) as factors. We then performed sex-specific multivariate general linear models (GLMs) on all other life-history traits (sex-specific lean weight and fat content, female RA and fecundity, male GSI, embryo lean weight and embryo fat content) with population as the fixed factor of interest. In the GLM on male life-history traits, only SL was included as covariate, while SL and embryonic stage of development served as covariates in the model on female life-history traits.

To evaluate the mode of maternal provisioning, we calculated the matrotrophy index (MI) using the slopes and intercepts from the regression analysis described below. The MI equals the estimated dry mass of the embryo at birth divided by the estimated dry mass of the ovum at fertilization (e.g., Reznick et al. 2002b, Riesch et al. 2011, 2013). If the eggs were fully provisioned by yolk before fertilization (lecithotrophy), then we would expect the embryos to lose 25%–40% of their dry mass during development (MI between 0.60 and 0.75; Scrimshaw 1945, Wourms 1981). On the other hand, in the case of continuous maternal provisioning after fertilization (matrotrophy), one would expect the embryos to lose less weight (MI between 0.75 and 1.00) or to even gain weight during development (MI > 1.00; e.g., Reznick et al. 2002b). Thus, maternal provisioning was evaluated by analyzing the relationship between log-transformed embryonic dry mass and stage of development by means of linear regression analysis (Reznick et al. 2002b, Riesch et al. 2011, 2013).

Results

In the ANOVA on body size, we found that sexes ($F_{1,60} = 109.70$, $P < 0.001$) and populations differed from one another ($F_{1,60} = 16.82$, $P < 0.001$), while the interaction sex-by-population was not significant ($F_{1,60} = 3.57$, P = 0.064). Male guppies had a smaller mean body size than females, and Pitch Lake guppies were smaller than guppies from the reference population (Fig. E.2A, E.2B).

In the multivariate GLM on male life-history traits we found a significant effect of both the covariate SL ($F_{3,24} = 52.52$, $P < 0.001$, Partial $\eta^2 = 0.868$) and the factor population ($F_{3,24} = 8.61$, $P < 0.001$, Partial $\eta^2 = 0.518$). Trait-specific *post-hoc* ANOVAs revealed that the effect of SL was due to a size-effect on lean weight ($F_{1,26} = 166.49$, $P < 0.001$), while fat content ($F_{1,26} = 0.02$, $P = 0.879$) and GSI ($F_{1,26} = 0.52$, $P = 0.478$) were not affected; however, all traits were significantly different between both populations (lean weight: $F_{1,26} = 11.87$, $P = 0.002$; fat content: $F_{1,26} = 7.73$, $P = 0.010$; GSI: $F_{1,26} = 7.93$, $P = 0.009$; Fig. E.2A).

In the multivariate GLM on female life-history traits we found significant effects of the covariates SL ($F_{6,26} = 235.30$, $P < 0.001$, Partial $\eta^2 = 0.982$) and embryonic stage of development ($F_{6,26} = 5.90$, $P = 0.001$, Partial $\eta^2 = 0.576$), as well as the factor population ($F_{6,26} = 5.82$, $P = 0.001$, Partial $\eta^2 = 0.573$). *Post-hoc* ANOVAs revealed that SL had a significant effect on lean weight ($F_{1,31} = 1587.96$, $P < 0.001$), fecundity ($F_{1,31} = 18.32$, $P < 0.001$)

and embryo lean weight ($F_{1, 31} = 15.14$, $P < 0.001$); whereas fat content ($F_{1, 31} = 0.17$, $P = 0.686$), embryo fat content ($F_{1, 31} = 0.68$, $P = 0.416$) and RA ($F_{1, 31} = 0.49$, $P = 0.491$) were not affected (Fig. E.2B). Moreover, stage had a significant effect on fecundity ($F_{1, 31} = 13.78$, $P = 0.001$), embryo lean weight ($F_{1, 31} = 10.30$, $P = 0.003$) and RA ($F_{1, 31} = 25.09$, $P < 0.001$), while it did not affect the other traits (lean weight: $F_{1, 31} = 0.00$, $P = 0.986$; fat content: $F_{1, 31} = 0.19$, $P = 0.667$; embryo fat content: $F_{1, 31} = 0.11$, $P = 0.740$; Fig. E.2C). On the population level, we found significant differences in lean weight ($F_{1, 31} = 29.30$, $P < 0.001$), embryo lean weight ($F_{1, 31} = 12.19$, $P = 0.001$) and RA ($F_{1, 31} = 4.35$, $P = 0.045$); however, there were no significant differences in fat content ($F_{1, 31} = 3.27$, $P = 0.080$), embryo fat content ($F_{1, 31} = 0.36$, $P = 0.552$) or fecundity ($F_{1, 31} = 3.80$, $P = 0.060$; Fig. E.2B).

According to the MI, both populations were characterized by a lecithotrophic provisioning strategy, and guppies from the reference site produced offspring nearly twice as heavy at birth as Pitch Lake guppies (Table E.1; Fig. E.2D).

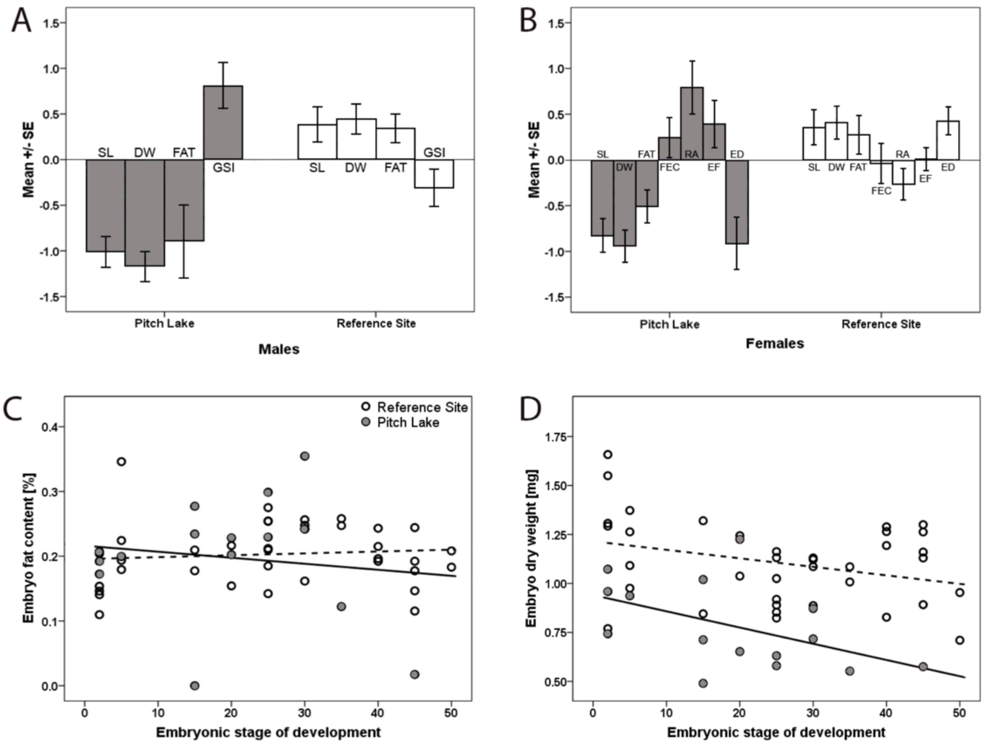

Figure E.2. Mean *z*-transformed life-history traits of male (A) and female (B) guppies. SL: standard length, DW: dry weight, FAT: fat content, GSI: gonadosomatic index, FEC: fecundity, RA: reproductive allocation, EF: embryo fat content, and ED: embryo dry weight. Scatter plots depicting (C) embryo fat content and (D) embryo dry weight during the course of embryo development.

Discussion

Overall, life histories quantified for both guppy populations were within the range of those previously reported for Trinidadian guppies, although some trait values in Pitch Lake guppies, such as estimated offspring size at birth and MI, were on the lower end of those previously reported (e.g., Reznick and Bryga 1987, Reznick et al. 1996, Pires et al. 2010).

We largely found our *a priori* predictions confirmed: guppies from the Pitch Lake were smaller (SL), had lower lean weight and fat content, and higher GSI (males) and RA (females) compared to guppies from the reference population. Furthermore, females from the Pitch Lake produced more and smaller offspring than the reference-site females. This is congruent with a scenario of higher mortality across age classes in the Pitch Lake, similar to what has been reported in several river systems in southern Trinidad by Rolshausen et al. (2015) who investigated guppy responses to human-induced crude-oil PAH (polycyclic aromatic hydrocarbon) pollution.

In agreement with previous studies, our environmental data indicated that the Pitch Lake was highly acidic, and in at least some places had higher water temperatures compared to our reference site. Previous work on livebearing fishes suggested that lower pH should result in increased fecundity, lean weight and body size (e.g., Riesch et al. 2015a), while higher water temperatures should also result in higher fecundity and greater investment into reproduction (e.g., Vondracek et al. 1988, McManus and Travis 1998). Our results are in partial agreement with this, as the low pH and slightly higher water temperatures of the Pitch Lake were associated with higher fecundity and greater investment into reproduction, but patterns of body size and lean weight were opposite to those associated with pH in a previous study on several species of *Gambusia* (Riesch et al. 2015a). However, we currently do not know if these differences in pH and temperature between the sites are temporally stable or unique to our specific time of sampling.

Interestingly, we did not find any evidence for selection for larger offspring size coupled with a reduced fecundity, as is often the case in hydrogen sulphide (H_2S) toxic waters, even though Richardson (1912) reported the presence of traces of H_2S also in the Pitch Lake. One possible explanation for this is that the concentration of H_2S is too low to elicit increased offspring size (for a similar scenario see for instance Riesch et al.

2016). Alternatively, other toxins in the water or other selective agents might largely select for increased fecundity coupled with smaller offspring size, and this simply overrides H_2S-induced selection.

One such environmental factor that might differ between the Pitch Lake and our reference site, and might camouflage phenotypic responses to toxin-induced selection, is predation. Personal observations during sampling and previous studies (Mohammed et al. 2010, Schelkle et al. 2012) suggest that the Pitch Lake is likely to be a high predation environment due to a high abundance of piscivorous birds (among them kingfishers, herons, egrets, yellow-billed tern, *Sternula superciliaris*, and black skimmer, *Rynchops niger*; Fig. E.1C), the presence of several cichlids, Guyana leaffish, as well as a relatively high density of piscivorous *Rivulus hartii*. In contrast, guppies were the only fish caught and observed at the reference site (D. Bierbach and M. Schartl, personal observation). In high-predation sites, guppies are usually characterized by high levels of extrinsic mortality, which also translates into *r*-selected phenotypes. In fact, Pitch Lake guppies exhibited all life-history traits typical for poeciliids in high-predation environments: small offspring size coupled with high fecundity, high investment into reproduction, smaller body size, and reduced body fat (*P. reticulata*, Reznick and Endler 1982; *Brachyraphis rabdophora*, Regan, 1908, Johnson and Belk 2001; *Gambusia hubbsi*, Poey, 1854, Riesch et al. 2013). Therefore, while our results are largely in line with our a priori predictions regarding bitumen-based toxicity (see above), they are also congruent with this interpretation of selection due to predation. Future studies should attempt to more clearly separate the different effects that predation, pH, water temperature, productivity, and food availability, might have on guppies from Pitch Lake and other sites.

Oil pollution, usually caused by human activities, is an extremely detrimental environmental factor for aquatic habitats (Neff 1987). Studying the effect of oil pollution on population dynamics of aquatic organisms has therefore important consequences for the conservation of aquatic environments (Hamilton et al. 2017). Trinidad in particular has a long history of oil exploitation that causes human-induced oil pollution in many rivers (Rolshausen et al. 2015). In this regard, guppies in the Pitch Lake present a unique study opportunity, since they inhabit a very peculiar place that experiences natural oil-pollution. Future studies should test if Pitch Lake guppies might even be locally adapted to this harsh environment.

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