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Grandparental effects in marine sticklebacks: transgenerational plasticity across multiple generations

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Abstract

Nongenetic inheritance mechanisms such as transgenerational plasticity (TGP) can buffer populations against rapid environmental change such as ocean warming. Yet, little is known about how long these effects persist and whether they are cumulative over generations. Here, we tested for adaptive TGP in response to simulated ocean warming across parental and grandparental generations of marine sticklebacks. Grandparents were acclimated for two months during reproductive conditioning, whereas parents experienced developmental acclimation, allowing us to compare the fitness consequences of short-term vs. prolonged exposure to elevated temperature across multiple generations. We found that reproductive output of F1 adults was primarily determined by maternal developmental temperature, but carry-over effects from grandparental acclimation environments resulted in cumulative negative effects of elevated temperature on hatching success. In very early stages of growth, F2 offspring reached larger sizes in their respective paternal and grandparental environment down the paternal line, suggesting that other factors than just the paternal genome may be transferred between generations. In later growth stages, maternal and maternal granddam environments strongly influenced offspring body size, but in opposing directions, indicating that the mechanism(s) underlying the transfer of environmental information may have differed between acute and developmental acclimation experienced by the two generations. Taken together, our results suggest that the fitness consequences of parental and grandparental TGP are highly context dependent, but will play an important role in mediating some of the impacts of rapid climate change in this system.

Introduction

Along with migration, within-generation phenotypic plasticity and genetic adaptation, transgenerational plasticity (TGP) is now recognized as a highly effective mechanism by which organisms can respond to rapid climate change (Bonduriansky *et al.*, 2012; Salinas *et al.*, 2013). Transgenerational plasticity is a type of nongenetic inheritance whereby the environment experienced by parents influences offspring reaction norms (different phenotypes expressed by the same genotype in different environments) and is manifest as a parent environment by offspring environment interaction (Mousseau & Fox, 1998). One advantage of TGP is speed – TGP is an inherited, fast, phenotypic response mechanism that can buffer populations against impacts of climate change currently experienced by the parent and provide time for genetic adaptation to catch up (Chevin *et al.*, 2010; Bonduriansky *et al.*, 2012). Another benefit of TGP is that it is often (but not always) adaptive (Marshall & Uller, 2007; Räsänen & Kruuk, 2007). For example, parents in stressful environments prime offspring for predicted stressful conditions, resulting in offspring that perform better under stress in comparison with offspring whose parents did not prime them (Herman *et al.*, 2012 and references therein).

Evidence for TGP is taxonomically diverse and spans a great array of traits (reviewed in Salinas *et al.*, 2013).

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Yet, little is known about how long these effects last. In many plant taxa and several animal species, environmental parental effects have been shown to persist for several generations (Roach & Wulff, 1987; Bernardo, 1996), but such grandparent (and beyond) environmental effects have rarely been investigated in non-model species and wild populations (but see Herman *et al.*, 2012; Lock, 2012). Grandparent effects can result from environmental parental effects that carry-over across more than one generation (e.g. detected as grandparent environment main effect in an ANOVA), grandparental TGP (grandparent environment by offspring environment interaction), or genetic parental effects (*sensu* genetic maternal effects in Reznick, 1981). In Reznick's (1981) example of grandfather effects in mosquito fish, he found a significant dam component of additive genetic variance for offspring size, but no sire component. Male effects were only significant in the F2 generation, which he interpreted as a cross-generational genetic maternal effect (i.e. maternal grandfather effect; Heath & Blouw, 1998).

Examples of parental TGP in response to changing environments are accumulating quickly, especially for marine species. In the marine realm, TGP in response to ocean acidification and warming sea surface temperatures has recently been shown in numerous invertebrates (Burgess & Marshall, 2011; Parker *et al.*, 2012; Vehmaa *et al.*, 2012; Kelly *et al.*, 2013) and several species of fish (Donelson *et al.*, 2012; Miller *et al.*, 2012; Salinas & Munch, 2012; Shama *et al.*, 2014), highlighting TGP as an important mechanism in marine systems to buffer populations against environmental stressors associated with rapid climate change (Munday *et al.*, 2013; Reusch, 2013; Sunday *et al.*, 2014). However, to date, few published studies have explicitly tested for grandparent effects or grandparental TGP in marine species (but see Donelson *et al.*, 2012). Hence, we have little knowledge about how many generations are required for the nongenetic effects of the environment to be removed (Salinas *et al.*, 2013), or if the effects are cumulative over generations (Herman *et al.*, 2012). Moreover, it may be that TGP will only be fully expressed if the parental generation has also had the opportunity for developmental acclimation (Donelson *et al.*, 2012; Burton & Metcalfe, 2014), and experiments covering at least two generations will be necessary to detect the full plasticity response available (Munday *et al.*, 2013).

In this study, we tested for adaptive TGP in response to simulated ocean warming across parental and grandparental generations of a marine population of three-spine stickleback, *Gasterosteus aculeatus* (Linnaeus, 1758). Previous studies of this population found that elevated summer water temperatures simulated in accordance with a 2100 scenario (Sheppard, 2004) had detrimental effects on growth (Schade *et al.*, 2014) and development (Ramler *et al.*, 2014). Yet, when parents were acclimated to elevated temperature during reproductive conditioning, offspring reached a larger size in the warmer (stressful) environment, and this parental TGP was driven solely by maternal acclimation temperature (Shama *et al.*, 2014). Here, we extend our investigation to the F2 generation to test for grandparental TGP and were particularly interested if parental (in this case maternal) TGP benefits on offspring size persist for more than one generation, and if these effects are cumulative. We focus on the influence of elevated temperature during developmental acclimation on reproductive output traits of the parental (F1) generation and growth trajectories of juvenile F2 offspring, allowing us to compare the fitness consequences of parental vs. grandparental environments on early life stages where detrimental effects of ocean warming seem to be strongest (Sunday *et al.*, 2014).

Materials and methods

Fish crosses

Grandparent fish originated from an oceanic stickleback population in the Sylt-Rømø Bight, Germany (55°05′N, 8°41′E). Wild adult fish were caught by trawling in February 2012 and held at two experimental acclimation temperatures (17 °C and 21 °C) for two months prior to producing F1 crosses. In May 2012, we produced pure crosses within and reciprocal crosses between acclimation temperatures and reared F1 offspring at both temperatures (see Shama *et al.*, 2014 for details). F1 families were reared individually for the first 60 days, after which they were pooled within each sire–dam–offspring temperature combination group (8 groups in total; see crossing design Table 1a). Each group was then divided amongst 2–4 replicate 25 L aquaria to reach a final density of 25–30 fish per aquaria (i.e. the number of fish per group ranged from 50 to 100). Groups were maintained at their offspring rearing temperature (e.g. four groups at 17 °C, four groups at 21 °C) until they reached adulthood. Fish were fed daily with chironomid larvae *ad libitum*.

F2 crosses were performed over a three-week period in March 2013 to produce full-sibling families in 16 temperature combination groups (Table 1b). We produced pure crosses (parent and grandparent temperatures the same) and mixed reciprocal crosses (parent and grandparent temperatures differed). Briefly, crosses were performed by strip spawning, and eggs were divided into halves in a Petri dish containing moist paper towel. Female size was measured as standard length (± 1 mm). We killed a male in an excess of MS-222 and removed the testes. Testes were crushed in isotonic nonactivating medium (Fauvel *et al.*, 1999), and sperm mobility was checked visually under a stereomicroscope before the solution was applied to eggs. Fertilized eggs were left for 30 min. before assigning them to

Table 1 Crossing designs for *Gasterosteus aculeatus* (a) F1 adults used as parental fish and (b) F2 offspring families. F1 crosses are shown as male (grandsire) °C × female (granddam) °C (e.g. 17 × 17) reared at either 17 °C or 21 °C (parental temperature). Temperature combination groups are depicted as G1, G2, etc. F2 crosses were also reared at 17 °C and 21 °C (not shown). The number of F2 families produced in each cross combination is indicated.

(a) F1 Group	Parental °C	Grandparental °C
G1	17	17 × 17
G2	17	17 × 21
G3	17	21 × 17
G4	17	21 × 21
G5	21	17 × 17
G6	21	17 × 21
G7	21	21 × 17
G8	21	21 × 21

(b) F2		Female							
Male		G1	G2	G3	G4	G5	G6	G7	G8
	G1	$n = 3$							$n = 0$
	G2		$n = 3$					$n = 3$	
	G3			$n = 2$			$n = 4$		
	G4		$n = 1$		$n = 2$	$n = 6$			
	G5				$n = 2$	$n = 3$			
	G6			$n = 1$			$n = 1$		
	G7		$n = 4$			$n = 3$		$n = 1$	
	G8	$n = 0$							$n = 0$

temperature treatments. As there were no sexually mature fish in group 8 (G8) during this time, we were unable to produce cross combinations that included that group, for example. no G8×G8, G1×G8 or G8×G1 (Table 1b). We also had difficulty obtaining good quality sperm from G6 males (LNS Shama *pers. obs.*), hence, the low number of crosses from that group. To increase sample size in some parental or grandparental temperature combinations, we produced additional crosses from groups with sexually mature fish, for example between G4×G2 and G7×G5. In total, we produced 39 F2 families from 15 temperature combination groups, with a range of $n = 15$ to $n = 24$ families per parental (sire/dam) temperature and $n = 11$ to $n = 28$ families per grandparental temperature (Table 1b). Egg clutches from each family were split and reared at 17 °C and 21 °C ($n = 78$ split clutches/families in total).

Egg traits and offspring body size

Each split clutch was photographed under a dissecting microscope for digital analyses of egg size and clutch size (using LEICA QWIN imaging software, Leica Microsystems Imaging Solutions Ltd., Cambridge, UK). Egg size in each family was estimated by measuring the diameter (± 0.01 mm) of ten eggs from each clutch. The ten measured eggs were chosen based on the clarity of their outer perimeter in the photograph, that is edges not distorted by contact with neighbouring eggs. Clutch size was estimated as the total number of eggs per female. Each split clutch was placed individually in a 1-L glass beaker containing filtered seawater and an air supply. Beakers were placed into water baths heated with aquarium heaters at either 17 °C or 21 °C. Hatching success was estimated as the proportion of hatchlings from each split clutch (no. hatchlings/no. eggs). Hatchlings were held in beakers for the first 30 days. Hatchling densities were reduced to approx. 10 offspring per beaker at 14 days post-hatch (note: clutches with max. 15 hatchlings were not reduced to 10 and 'rests' due to space constraints). Water was changed in the beakers every week. At 30 days post-hatch, 10 randomly chosen offspring from each split clutch were photographed under a dissecting microscope for digital analysis of body size (standard length ± 0.01 mm; using Leica QWin). At this point, the 10 offspring were transferred to a 2-L aquarium connected to a flow-through seawater system set at either 17 °C or 21 °C for another 60 days. At 60 and 90 days post-hatch, standard length was again measured on the 10 offspring per family by digital photography. Throughout the experiment, juvenile fish were fed daily with live *Artemia* sp. nauplii *ad libitum*.

Data analyses

We fitted generalized linear mixed models (GLMM) using ANOVA for significance testing and concentrated on traits related to reproductive output of F1 parental fish (egg size, clutch size and hatching success) and F2

offspring body size as decisive components of fitness. For body size analyses, we accounted for differences in fish densities by including initial hatchling densities in the beakers (0–14 days) in the 30-day model, and current density in the 60 and 90 day models, that is, to account for any families that had fewer (or more) than 10 individuals and for any mortality during the experiment. As we did not have a complete full-factorial design (due to missing G8 combinations), we did not include any parent temperature x grandparent temperature interaction terms in our models. We also did not include egg size as a covariate as egg size is an intermediate variable that may have been affected by temperature treatments in the F0 and F1 generations. We modelled egg traits and offspring body size at 30, 60 and 90 days with Gaussian errors and family as a random effect using the *lme* function from the R package 'nlme'. Hatching success was modelled with binomial errors, family as a random effect and an individual-level random effect to account for overdispersion using *glmer* implemented in the R package 'lme4'. We fitted all models using individuals, that is, individual eggs for analyses of hatching success and egg size, and individual fish for offspring body size. For graphical representation of offspring body size, we chose to display residuals of body size (standard length corrected for density). Residuals were calculated using linear models of body size ~ density. All analyses were run in the R statistical environment (R Development Core Team, 2011).

Results

Egg traits

Egg size was significantly influenced by dam temperature and clutch size (Fig. 1). Females that developed at 21 °C produced smaller eggs ($F_{1,29} = 17.031$; $P < 0.001$) but larger clutches ($F_{1,29} = 7.616$; $P = 0.010$) than females that developed at 17 °C. Clutch size traded off with egg size ($F_{1,29} = 6.202$; $P = 0.019$; Fig. 1) and was also influenced by paternal grandsire (PGS) temperature, with smaller clutches produced when PGSs were acclimated to 21 °C ($F_{1,29} = 4.690$; $P = 0.039$). Paternal granddam (PGD) and maternal grandparent (MGS and MGD) acclimation temperatures, as well as their interactions with other model terms, did not significantly influence egg size or clutch size (all $P > 0.05$). Female size did not differ between developmental temperatures ($F_{1,37} = 1.419$; $P = 0.241$), and there were no significant effects of female size on egg size ($F_{1,29} = 0.247$; $P = 0.623$) or clutch size ($F_{1,29} = 1.238$; $P = 0.275$). In other words, egg size was predominantly determined by maternal environment, and there was an inverse relationship between egg size and clutch size, but only clutch size showed carry-over effects from grandparental environment.

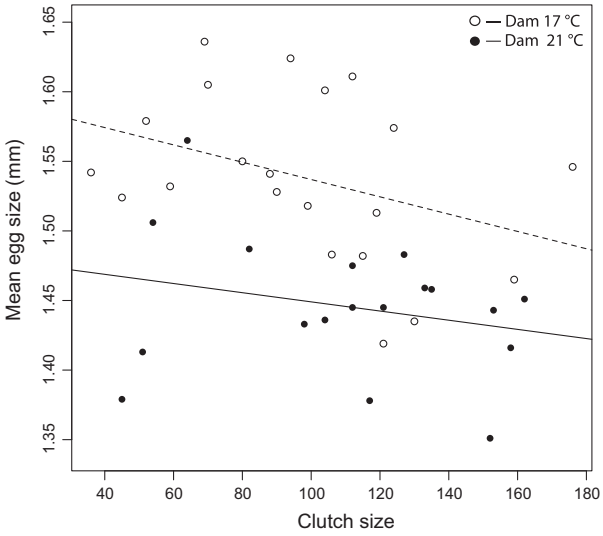

Fig. 1 Relationship between *Gasterosteus aculeatus* clutch size (total no. eggs per female) and mean egg size (mean diameter ± 0.01 mm of 10 eggs per female) for mothers that developed at 17 °C (open circles; dashed line) and 21 °C (closed circles; solid line).

Hatching success

Of the 39 families produced (and then split by temperature), 33 hatched at 17 °C and 28 hatched at 21 °C. Three clutches that failed at 21 °C were from 21 °C females, and two failed clutches were from 17 °C females. Six families failed to hatch at both temperatures due to problems with aeration. Hatching success was significantly influenced by offspring temperature, both parental temperatures, PGS and MGD temperature, and two 3-way interactions between offspring and parent/grandparent temperatures (Table 2; Fig. 2). Hatching success was in general lower at 21 °C than at 17 °C, and eggs from 21 °C dams, 21 °C sires and 21 °C paternal grandsires had lower hatching success than eggs with 17 °C in their parental/grandparental thermal history (Fig. 2), indicating cumulative negative effects of 21 °C sires down the paternal line. The 3-way interaction between offspring, PGS and MGD temperatures (Table 2) indicates grandparental TGP, but with positive TGP effects at 21 °C arising only from 21 °C MGDs that were mated with 17 °C PGSs (Fig. 2b).

Offspring body size

Density had significant effects on offspring body size (Table 3). Offspring in families with higher densities reached smaller body sizes. Density also differed between offspring temperatures at 60 ($F_{1,59} = 4.089$; $P = 0.048$) and 90 days ($F_{1,59} = 4.098$; $P = 0.048$). Mean density per family was 10.19 fish at 17 °C and

Table 2 Generalized linear mixed model (GLMM) showing the influence of offspring rearing temperature (offspring °C), parental developmental temperature (sire °C, dam °C) and grandparental acclimation temperature (PGS °C, MGS °C, PGD °C, MGD °C) on *Gasterosteus aculeatus* hatching success.

Random effects	Variance		Std. Dev.
Family (intercept)	3.833		1.958
Hatch (intercept)	0.008		0.088
Fixed effects	**Estimate**	**Std. Error**	**z value**	**Pr (>|z|)**
(Intercept)	6.181	4.589	1.347	0.178
Female size	−0.113	0.096	−1.178	0.239
Offspring °C	−0.487	0.029	−16.532	**< 0.001**
Sire °C	2.368	1.053	2.448	**0.025**
Dam °C	−2.677	0.954	−2.805	**0.005**
PGS °C	−1.391	1.353	−1.028	0.304
MGS °C	0.651	1.522	0.428	0.669
PGD °C	2.735	1.420	1.926	0.054
MGD °C	−3.139	1.711	−1.835	0.067
Offspring × Sire °C	−0.575	0.040	−14.215	**< 0.001**
Offspring × Dam °C	−1.089	0.043	−25.187	**< 0.001**
Sire × Dam °C	−0.496	1.447	−0.343	0.732
Offspring × PGS °C	0.660	0.060	11.034	**< 0.001**
Offspring × MGS °C	0.061	0.064	0.954	0.340
Offspring × PGD °C	0.054	0.066	0.809	0.419
Offspring × MGD °C	0.247	0.072	3.415	**0.001**
PGS × MGD °C	0.276	2.615	0.105	0.916
MGS × PGD °C	−2.929	2.692	−1.088	0.277
Offspring × Sire × Dam °C	1.028	0.067	15.412	**< 0.001**
Offspring × PGS × MGD °C	−0.762	0.120	−6.373	**< 0.001**
Offspring × MGS PGD °C	0.212	0.122	1.743	0.081

Model fit with individual-level variation (accounting for overdispersion) by the Laplace approximation and a binomial error distribution using *glmer* implemented in the R package lme4 (R Development Core Team, 2011). Std. Dev. and Std. Error indicate standard deviation and standard error, respectively. Significant terms are highlighted in bold. PGS, paternal grandsire; MGS, maternal grandsire; PGD, paternal granddam; MGD, maternal granddam.

9.69 fish at 21 °C, but the range of densities per family in the different temperatures overlapped (Fig. S1). Differences in densities between temperatures likely stem from differences in hatching success. At the start of the experiment, there were more eggs at 21 °C than 17 °C (n = 2328 vs. n = 1700). However, as hatching success was greater at 17 °C than 21 °C, there were fewer 21 °C offspring in the experiment (n = 213 at 21 °C vs. n = 301 at 17 °C). Nevertheless, any growth advantages of lower densities at 21 °C would only dampen the size differences found between temperatures (see below). Moreover, density x offspring temperature interactions were not significant at either 60 ($F_{1,57}$ = 0.800; P = 0.375) or 90 days ($F_{1,57}$ = 1.607; P = 0.210), indicating that any potential effects of density on offspring body size were the same in both temperatures (Fig. S1).

Both parental and grandparental thermal environments significantly influenced offspring body size (Table 3). At 30 days, interactions between offspring and sire temperatures on the one hand, and two 3-way interactions between offspring and grandparental temperatures on the other, indicate paternal as well as grandparental TGP (Table 3). Transgenerational effects were positive in both cases, as offspring reached larger sizes when reared in their paternal and grandparental environments (after controlling for density effects). Specifically, at 17 °C, offspring of 17 °C fathers were larger than offspring of 21 °C fathers, and at 21 °C, offspring of 21 °C fathers were larger than offspring of 17 °C fathers (Fig. 3a). Similarly, at 21 °C, offspring reached the largest sizes when both grandparents were acclimated to 21 °C (Fig. 3b,c), especially down the maternal grandparent line. Maternal effects were, however, negative, as depicted by smaller offspring sizes when mothers were acclimated to 21 °C (Fig. 3a). The interaction between offspring and dam temperature (Table 3) likely reflects the substantial size difference between offspring of 17 × 17 parents reared at 17 °C vs. 21 °C (Fig. 3a).

At 60 days, dam temperature again had a significant effect on body size – offspring of 21 °C mothers were smaller than those from 17 °C mothers at both rearing temperatures (Fig. 3d). The 3-way interaction between offspring, MGS and PGD °C temperatures was also detected at 60 days (Table 3), but the effects were now negative, with offspring showing smaller sizes when MGSs and PGDs were acclimated to 21 °C (Fig. 3e,f). That is, positive TGP effects at 21 °C attributable to fathers and paternal grandparents seen at 30 days were no longer present. Yet, offspring were (relatively) larger at 21 °C when MGDs were acclimated to 21 °C (Fig. 3f, i), indicating positive two-generation maternal effects (Table 3). By 90 days, differences in mean offspring size between maternal temperatures were even more pronounced (Table 3; Fig. 3g). A significant 3-way interaction between offspring and parental temperatures (offspring x sire x dam; Table 3) likely reflects a stronger maternal influence at 17 °C but a stronger paternal influence at 21 °C in the 17 × 21 and 21 × 17 parental groups (Fig. 3g). Neither parental nor grandparental TGP was detected at 90 days. At all time points, mean offspring body size differed between rearing temperatures, with offspring reared at 17 °C reaching larger sizes than offspring reared at 21 °C (Table 3; Fig. 3a,d,g).

Discussion

Our study illustrates the influence of both grandparental and parental thermal environments on key fitness traits of marine sticklebacks. Reproductive output of F1 adults was primarily determined by maternal developmental temperature, but carry-over effects from

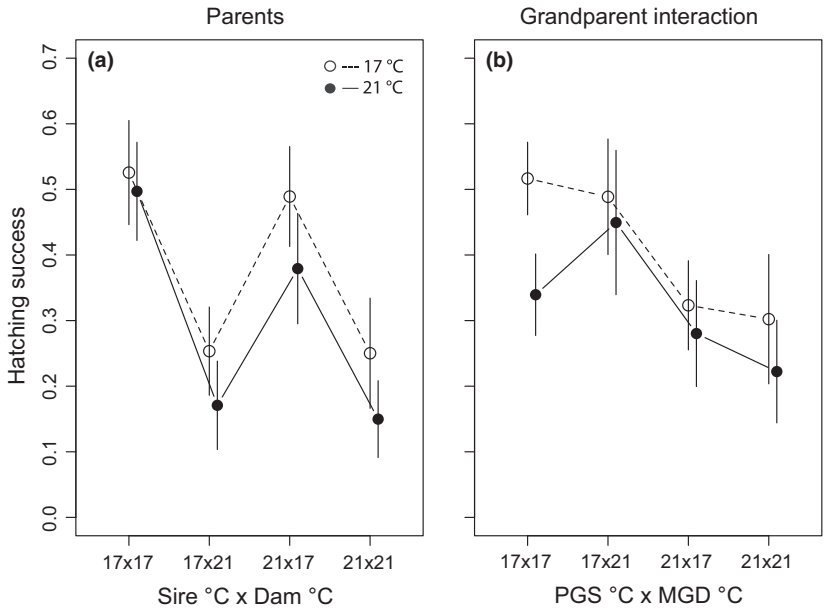

Fig. 2 Hatching success (shown as hatching proportion) of *Gasterosteus aculeatus* clutches produced from crosses between (a) parental and (b) grandparental temperatures reared at 17 °C (open circles) and 21 °C (closed circles). Parental temperatures are shown as male (sire) temperature x female (dam) temperature. Grandparent interaction shows the interaction between PGS (paternal grandsire) and MGD (maternal granddam) temperatures. Points depict means (±SE) for all families within a temperature combination group. Lines join parental or grandparental temperature combinations.

grandparental acclimation environments resulted in cumulative negative effects of elevated temperature across generations on hatching success. Body size of juvenile F2 offspring benefitted from both paternal and grandparental TGP in very early stages of growth. In later stages, maternal and MGD environments influenced offspring body size, but in opposing directions, indicating that positive grandmother effects were still present albeit diluted after two generations. Taken together, our results suggest that both parental and grandparental TGP will play a role in mediating some of the impacts of climate change in this system, but that parental TGP may represent a more immediate buffer to environmental conditions prevailing in the population. Moreover, the transfer of environmental information across multiple generations may rely on more than one nongenetic pathway that differs with acute or developmental acclimation in the parental or grandparental generation.

Egg size plasticity and hatching success

Stickleback mothers allocated resources to eggs differently depending on the thermal environment they experienced during development. In line with other findings of egg size plasticity in response to oviposition temperature (Bownds *et al.*, 2010; Liefting *et al.*, 2010), females produced larger, but fewer eggs in the colder (ambient) environment, and smaller, but more eggs at elevated temperature. Variation in initial size can be propagated through an individual's life (Mousseau & Fox, 1998), but whether this size variation is adaptive or not depends on the relationship between offspring size and performance in the respective environment (Kaplan, 1992; Bownds *et al.*, 2010; Marshall *et al.*, 2010). Although high temperatures have been shown to lead to greater incubation failure and mortality in sticklebacks (Hopkins *et al.*, 2011), smaller eggs at elevated temperature may be an advantage due to their lower oxygen demands (Kolm & Ahnesjö, 2005). Still, size-related oxygen demands will depend on the proportion of yolk vs. higher respiring embryo tissue in eggs at different temperatures (Hendry & Day, 2003), which remains to be tested for sticklebacks.

Reproductive output varied with maternal temperature independent of female size, suggesting that egg size plasticity was not simply due to physiological constraints (Heath & Blouw, 1998), but that different environments elicit selection for different-sized offspring (Bownds *et al.*, 2010; Marshall *et al.*, 2010). If females allocated egg resources to increase offspring fitness in predicted future environments, then egg size plasticity in response to maternal temperature is a classic example of an anticipatory maternal effect or adaptive TGP (Mousseau & Fox, 1998; Marshall & Uller, 2007; Räsänen & Kruuk, 2007). Alternatively, females that developed at elevated temperature produced offspring of smaller size in favour of fecundity (selfish maternal effect *sensu* Marshall & Uller, 2007), thereby maximizing their own fitness over offspring fitness in the stressful environment (Kirkpatrick & Lande, 1989). Interestingly, we did not find egg size plasticity in the grandparental fish used to produce the F1 generation, that is, wild caught fish that had been acclimated for two months during reproductive conditioning (Shama *et al.*, 2014). Reproductive plasticity was only seen when the parental generation had the opportunity for acclimation during all developmental stages (Donelson

Table 3 Linear mixed effects models for *Gasterosteus aculeatus* body size at 30, 60 and 90 days post-hatch depicting the influence of offspring rearing temperature (offspring °C), parental developmental temperatures (sire °C, dam °C) and grandparental acclimation temperatures (PGS °C, PGD °C, MGS °C and MGD °C).

	Size 30 days			Size 60 days			Size 90 days		
	denDF	*F*	*P*	denDF	*F*	*P*	denDF	*F*	*P*
Intercept	470	8999.121	**< 0.001**	463	25173.835	**< 0.001**	462	29831.273	**< 0.001**
Offspring environment effects									
Density	470	0.011	0.917	463	79.943	**< 0.001**	462	87.403	**< 0.001**
Female size	22	0.019	0.890	22	0.270	0.608	22	0.344	0.563
Offspring °C	470	48.244	**< 0.001**	463	93.663	**< 0.001**	462	185.211	**< 0.001**
Parental environment effects									
Sire °C	22	2.838	0.106	22	0.113	0.740	22	0.245	0.625
Dam °C	22	9.576	**0.005**	22	15.142	**0.001**	22	10.265	**0.004**
Sire × Dam °C	22	3.998	0.058	22	2.189	0.153	22	7.554	**0.012**
Offspring × Sire °C	470	25.318	**< 0.001**	463	0.189	0.664	462	1.078	0.300
Offspring × Dam °C	470	36.640	**< 0.001**	463	0.145	0.704	462	0.690	0.407
Offspring × Sire × Dam °C	470	0.559	0.455	463	0.003	0.954	462	4.439	**0.036**
Grandparental environment effects									
PGS °C	22	0.717	0.406	22	0.787	0.385	22	0.171	0.684
MGS °C	22	0.068	0.797	22	2.287	0.145	22	0.968	0.336
PGD °C	22	0.515	0.481	22	0.655	0.427	22	0.602	0.446
MGD °C	22	1.397	0.250	22	7.217	**0.014**	22	3.026	0.096
PGS × MGD °C	22	0.011	0.919	22	3.106	0.092	22	2.362	0.139
MGS × PGD °C	22	0.003	0.954	22	1.769	0.197	22	0.004	0.952
Offspring × PGS °C	470	8.776	**0.003**	463	1.547	0.214	462	0.164	0.686
Offspring × MGS °C	470	0.001	0.978	463	0.277	0.599	462	2.194	0.139
Offspring × PGD °C	470	15.251	**< 0.001**	463	6.956	**0.009**	462	0.183	0.669
Offspring × MGD °C	470	12.710	**< 0.001**	463	3.656	0.057	462	0.699	0.403
Offspring × PGS × MGD °C	470	12.223	**< 0.001**	463	2.208	0.138	462	0.858	0.355
Offspring × MGS × PGD °C	470	20.658	**< 0.001**	463	44.602	**< 0.001**	462	2.861	0.091

Size was measured as standard length (mm) at 30, 60 and 90 days post-hatch. Numerator degrees of freedom were 1 in all cases. denDF indicates denominator degrees of freedom. Significant terms are highlighted in bold. PGS, paternal grandsire; PGD, paternal granddam; MGS, maternal grandsire; MGD, maternal granddam.

et al., 2012; Burton & Metcalfe, 2014), and two generations were necessary to see the full egg size plasticity response (Munday *et al.*, 2013). Relating to this, although we found differences in mean egg size between maternal temperatures, we did not find significant differences in egg size variance (data not shown), suggesting that mothers were not using a bet-hedging strategy to spread the risk of incorrectly predicting future environments (Crean & Marshall, 2009; Morrongiello *et al.*, 2012). Our result may not be surprising given that mothers experienced the same temperature throughout their lives, and bet-hedging is a more likely outcome for this population when environmental conditions are unpredictable (LNS Shama & KM Wegner unpublished data).

Parental effects can either buffer offspring from environmental change or act as conduits whereby environmental stress in previous generations reduces productivity of later generations (Mousseau & Fox, 1998; but see Herman *et al.*, 2012). Our results for hatching success tend to point to the latter. Hatching success was lower at elevated temperature for all crossing groups, showing that 21 °C is a stressful hatching environment for this population. Female-mediated traits likely had a strong influence on hatching success. For instance, eggs from mothers that developed at elevated temperature were smaller, had lower hatching success and grew to become smaller offspring than eggs of 17 °C mothers. In addition, reduced sperm quality at higher temperature (Mehlis & Bakker, 2014) may also have played a role. Although we did not address sperm performance or fertilization success explicitly in our study, we did detect a negative effect of elevated paternal developmental temperature on hatching success. Hatching success was also lower when paternal grandsires were acclimated to 21 °C, indicating that negative consequences of elevated temperature on hatching success were cumulative across two generations down the paternal line. Our previous study of the F1 generation showed a similar pattern, with hatching success in general lower at 21 °C and even more so for eggs of 21 °C mothers (Shama *et al.*, 2014). Both studies suggest that selection gradients are steeper at elevated temperature, particularly for this life history stage (see also Hopkins *et al.*, 2011; Mehlis & Bakker, 2014), and that parental environment not only during reproductive conditioning

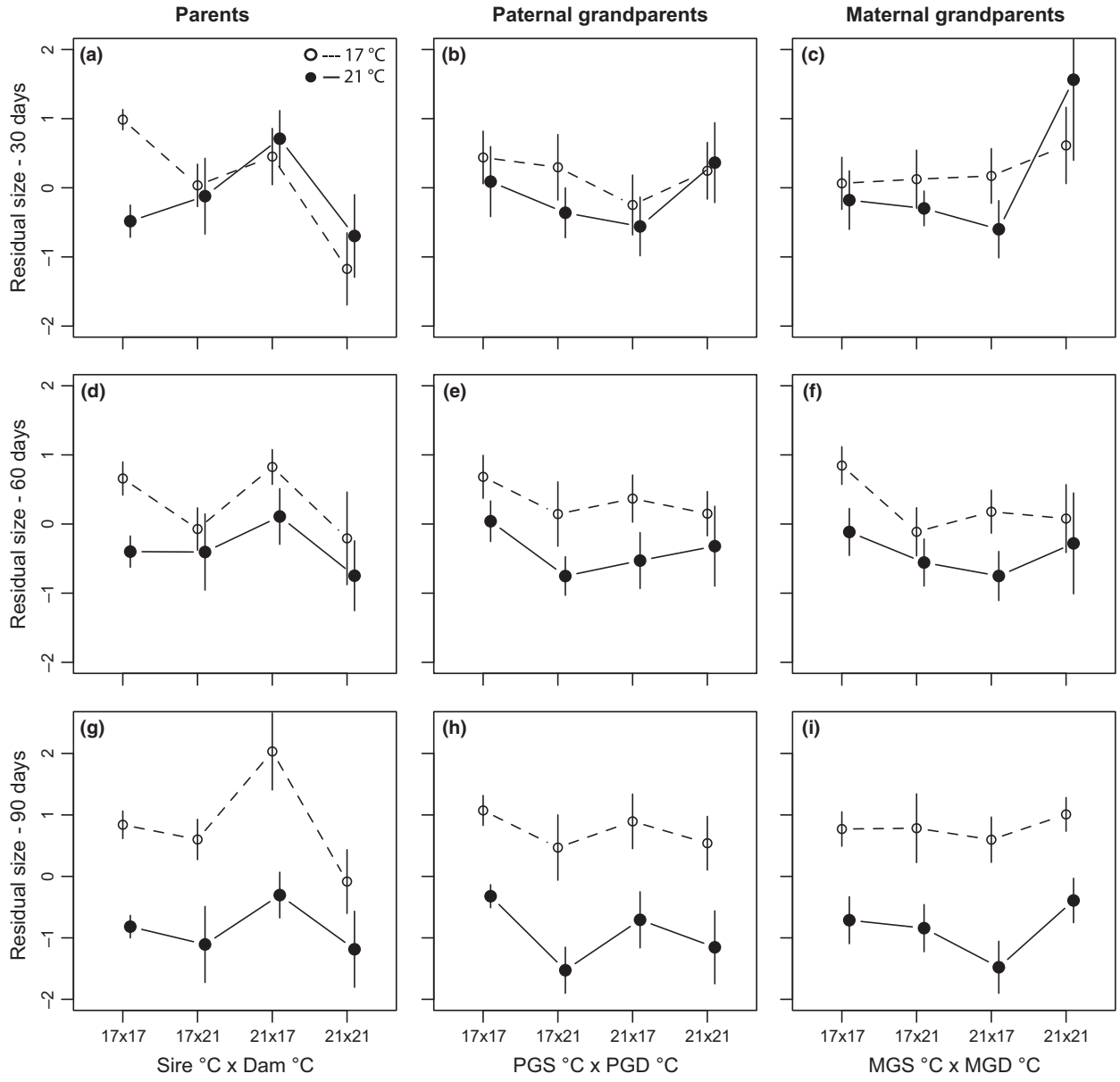

Fig. 3 *Gasterosteus aculeatus* offspring size corrected for density (shown as residuals of standard length) at (a–c) 30 days, (d–f) 60 days and (g–i) 90 days post-hatch for each parental and grandparental temperature combination and reared at 17 °C (open circles) and 21 °C (closed circles). Parental temperatures are shown as male (sire) temperature × female (dam) temperature, and grandparental temperatures are shown as grandsire °C × granddam °C (PGS = paternal grandsire, PGD = paternal granddam, MGS = maternal grandsire, MGD = maternal granddam). Points depict mean residuals (±SE) for all families within a temperature combination group. Lines join parental or grandparental temperature combinations.

but also in earlier developmental stages plays a key role in determining offspring survival at this point in time (Heath & Blouw, 1998; Burton & Metcalfe, 2014).

Transgenerational effects on body size

In the early stages of growth, offspring body size benefitted from both paternal and grandparental TGP. While maternal environment effects are pervasive in the literature (Mousseau & Fox, 1998; Marshall & Uller, 2007; Räsänen & Kruuk, 2007), recent evidence for paternal environmental effects raises the possibility that more than just the additive genetic effects of sires can influence offspring performance (Etterson & Galloway, 2002; Crean *et al.*, 2013). Here, we found that offspring grew better in their respective paternal environment, but

these effects were transient, not lasting beyond the first 30 days of growth. Acclimation to elevated temperature has been shown to influence sperm swimming performance in other fish species (Adriaenssens *et al.*, 2012), and plasticity of sperm phenotype may have contributed to the paternal TGP benefits seen for early offspring growth found here. It may also be that methylomes – DNA methylation patterns that can regulate gene expression – are paternally inherited, as has been recently shown in zebra fish (Jiang *et al.*, 2013; Potok *et al.*, 2013). Further support that paternal environment effects may play a role in alleviating some of the fitness (size) consequences associated with elevated temperature stems from interactions between paternal and maternal temperatures showing a stronger paternal influence on offspring size at 21 °C. Similarly, positive grandparental environmental effects down the paternal line indicate some compensation in offspring performance, although these benefits were also short-lived. In any case, our data suggest that more than just the paternal genome may be transferred between generations, with potential consequences for offspring performance in changing environments (see also Crean *et al.*, 2013).

The effects of parental environment on offspring phenotype are not always positive (Marshall & Uller, 2007; Räsänen & Kruuk, 2007; Marshall, 2008; Uller *et al.*, 2013). Here, we found that developmental acclimation of mothers at elevated temperature had negative effects on offspring body size – offspring were smaller when mothers developed at 21 °C. Still, positive maternal grandmother environment effects resulted in a (relative) body size increase at 21 °C when MGDs were acclimated to 21 °C. That these environmental effects across two generations influenced offspring body size in opposing directions argues against a strict 'conduit of stress between generations' scenario (Mousseau & Fox, 1998) for this life history stage, but rather, begs the question of whether the mechanism(s) underlying the transfer of environmental information differed in the two generations (Shea *et al.*, 2011). For instance, in the present study, egg size plasticity likely had a strong influence on resulting offspring body size, whereas in the previous (grandparental) generation, we suggested that mothers programmed offspring to perform better in their predicted future environment by adjusting mitochondrial respiration capacities (Shama *et al.*, 2014). While both mechanisms are forms of detection-based effects (*sensu* Shea *et al.*, 2011), egg size plasticity stems from resource allocation and may be considered a type of 'slow maternal programming' that develops based on lifetime or possibly early-life exposure (Donelson *et al.*, 2012; Burton & Metcalfe, 2014). Mitochondrial respiration plasticity, however, was seen after only two months of parental acclimation, perhaps resulting from epigenetic marks that affect genes associated with mitochondrial function and thermal tolerance, for example, by maternal transfer of mRNA (Shama *et al.*, 2014), and these 'fast-programming' positive grandmother effects may have persisted through to the F2 generation. Whether plasticity of mitochondrial respiration also occurs when mothers experience developmental acclimation requires additional studies using F2 offspring, but could help to determine whether the mechanisms underlying offspring phenotype plasticity differ depending on maternal experience, and whether one mechanism has overriding effects on the other.

Overall, offspring environment had the strongest and most persistent effects on body size. Offspring were smaller when reared at 21 °C vs. 17 °C, and this result is consistent with three previous studies of this population (Ramler *et al.*, 2014; Schade *et al.*, 2014; Shama *et al.*, 2014). Smaller offspring at elevated temperature is a common finding in climate change studies and points to a general response likely due to energetic restrictions (Daufresne *et al.*, 2009). Yet, as previously discussed for egg size, smaller body size at elevated temperature may be an advantage in terms of lower oxygen demands (Forster *et al.*, 2012), and lower growth rates at higher temperature are not always associated with reduced aerobic scope (Gräns *et al.*, 2014). Indeed, optimized rather than maximized metabolism at elevated temperature could generate a higher scope for growth if TGP benefits are present (Shama *et al.*, 2014) and may be an effective mechanism for mediating some of the impacts of ocean warming if populations experience a gradual increase in temperature over several generations (Miller *et al.*, 2012).

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Relationship between density and size (measured as standard length ± 0.01 mm) for *G. aculeatus* offspring reared at 17 °C (open symbols; dashed lines) and 21 °C (closed symbols; solid lines) at (a) 30 days, (b) 60 days and (c) 90 days post-hatch.

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