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Testing an ecophysiological mechanism of morphological plasticity in pupfish and its relevance to conservation efforts for endangered Devils Hole pupfish

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Summary

Imperiled species that have been translocated or established in captivity can show rapid alterations in morphology and behavior, but the proximate mechanisms of such phenotypic changes are rarely known. Devils Hole pupfish (*Cyprinodon diabolis*) are endemic to a single desert pool and are characterized by a small body, large head and eyes, and lack of pelvic fins. To lessen the risk of extinction, additional populations of *C. diabolis* were established in artificial refuges. Yet, pupfish in these refuges rapidly shifted to a larger body, smaller head and eyes, and greater body depth. Here we examined how food availability and temperature, which differ between these habitats, influence morphological development in closely related Amargosa River pupfish (*Cyprinodon nevadensis amargosae*). We were interested in knowing whether these environmental factors could developmentally shift Amargosa River pupfish toward the morphology typical of pupfish in Devil's Hole. By regulating food ration, we created groups of pupfish with low, medium and high growth rates. Pupfish with low growth showed proportionally larger head and eyes, smaller body depth, and reduction in pelvic fin development. Elevated temperature further inhibited pelvic fin development in all treatments. Pupfish in the low growth group also showed reduced levels of thyroid hormone, suggesting a possible physiological mechanism underlying these morphological changes. To test this mechanism further, pupfish were reared with goitrogens to pharmacologically inhibit endogenous thyroid hormone production. Pupfish given goitrogens developed larger heads and eyes, shallower bodies, and reduced pelvic fins. Taken together, our results suggest that changes in environmental factors affecting the growth and thyroid hormone status of juvenile pupfish may play a developmental role in generating the morphological differences between *C. diabolis* in Devil's Hole and the refuges. These findings illustrate the need to incorporate a mechanistic understanding of phenotypic plasticity into conservation strategies to preserve imperiled fishes.

Key words: development, phenotypic plasticity, morphology, captive breeding, translocation, ecophysiology, thyroid hormone, conservation.

Introduction

Recovery plans for conserving rare or endangered species often recommend that new populations be established to lessen the risk of extinction (Williams et al., 1988; Tear et al., 1993). For many imperiled taxa, individuals are brought into captivity to be bred for reintroduction or supplementation of declining wild populations, or to establish self-sustaining captive populations (Kleiman, 1989; Snyder et al., 1996; Wallace, 2000; Brown and Day, 2002). Other species with chronically small populations or highly restricted ranges may be translocated to new habitats (Minckley, 1995). Conditions in these new environments rarely mirror those of the native habitat, and new populations can rapidly change morphology, behavior, and physiology relative to those characteristics expressed in the natural population. Such unexpected changes in phenotype can hinder the survival and reproductive success of individuals raised in captivity and, in some cases, have led to genetic change (Philippart, 1995; Reisenbichler and Rubin, 1999). Understanding how these rapid changes in phenotype arise could help both to remedy and avoid unintended phenotypic alterations.

The potential benefits and problems of transferring individuals to novel habitats are illustrated by conservation efforts for the endangered Devils Hole pupfish (*Cyprinodon diabolis* Wales), a species that in many ways has served as an example for endangered species management (Deacon and Williams, 1991). Devils Hole pupfish are endemic to Devil's Hole, which is a small surface opening (~3 m wide by 20 m

length) located 15 m inside a rock fissure that leads to a deep groundwater aquifer (Soltz and Naiman, 1978). Devil's Hole has no surface outflow, and pupfish in this habitat forage and spawn only on a shallow limestone shelf at one edge (James, 1969). The pupfish in Devil's Hole are morphologically unique among pupfish species. They have a small body size, proportionally large head and eyes, and lack pelvic fins – characteristics that typify the juvenile life stage of other pupfish species (Wales, 1930; Miller, 1948). This suite of morphological characteristics suggests that the Devils Hole pupfish may be morphologically neotenous.

During the 1960s, ground water pumping caused the water level in Devil's Hole to fall, exposing the shallow rock shelf that provided the only spawning habitat (Deacon and Williams, 1991; Karam, 2005). This crisis instigated the construction of three artificial refuges – the Hoover Dam refuge, Point of Rocks refuge, and School Springs refuge (extinct since 2003) – to establish additional populations of *C. diabolis* and to provide a source of fish for reintroduction should the population in Devil's Hole go extinct (Sharpe et al., 1973; Baugh and Deacon, 1988; Karam, 2005). The artificial refuges were constructed specifically to emulate the ecological conditions in Devil's Hole (Sharpe et al., 1973; Williams, 1977). Nevertheless, only 5 years after their introduction, the morphology of *C. diabolis* in the Hoover Dam refuge was found to differ significantly from the Devil's Hole phenotype (Williams, 1977). Pupfish in the refuge had larger, deeper bodies and smaller head sizes than fish in Devil's Hole. In 2000, pupfish in the two other refuges were subsequently found to deviate morphologically along the same parameters, with 32% of fish in the School Springs refuge and 48% of fish in the Point of Rocks refuge exceeding the maximum reported body length of pupfish in Devil's Hole (Wilcox, 2001). These changes mark substantial morphological deviations from the phenotype of *C. diabolis* in its natural habitat.

Understanding how these morphological changes occurred is critically relevant to the successful management of *C. diabolis* (US Fish and Wildlife Service, 1990). Initial efforts examined whether the refuge populations had changed genetically from the Devil's Hole population. A molecular genetic comparison of these populations showed that refuge populations contain a subset of the alleles present in fish from Devil's Hole, suggesting that refuge populations have experienced founder effects or genetic drift (Wilcox, 2001). Even so, the genomes of *C. diabolis* are nearly identical at the allozyme, mtDNA and nuclear DNA levels (Echelle and Dowling, 1992; Echelle and Echelle, 1993; Duvernell and Turner, 1998; Duvernell and Turner, 1999; Martin and Wilcox, 2004), suggesting that genetic change may not fully explain the morphological shifts of the refuge populations (Wilcox, 2001).

What has not been tested previously is that the morphological differences between *C. diabolis* in the refuges and Devil's Hole might be caused by developmental plasticity – a developmental change in form or behavior caused by environmental conditions (West-Eberhard, 2003). Here, we explore this idea by examining how pupfish morphology responds developmentally to variation in environmental conditions. Given the crisis conservation status of *C. diabolis* in Devil's Hole (currently fewer than 50 individuals), we examined whether the morphological traits typical of pupfish in Devil's Hole could be experimentally induced in a surrogate sister species (Amargosa River pupfish, *C. n. amargosae*) by providing environmental conditions comparable to those in Devil's Hole. First, we regulated food ration to create experimental groups of Amargosa River pupfish with lower growth rates to approximately match the growth rate of *C. diabolis* in Devil's Hole (James, 1969). Pupfish in these treatments were then examined for differences in relative head and eye size, body depth and pelvic fin development. Recent work in other fish species has suggested that thyroid hormone in part mediates the morphological changes that occur as fish transition from larval to juvenile life stages (Brown, 1997; Trijuno et al., 2002). We thus used radioimmunoassay to measure whole-body levels of the thyroid hormone thyroxine (T_4) in pupfish from these food ration treatments. We also tested the hypothesis that morphological shifts between the refuge and Devil's Hole populations might be mediated in part by shifts in thyroid status by treating larval pupfish with goitrogens that pharmacologically block the endogenous production of thyroid hormones. We predicted that goitrogen treatments would cause *C. n. amargosae* to develop a morphology similar to *C. diabolis* in Devil's Hole.

Materials and methods

Animal collection

On 18 October 2001, male and female Amargosa River pupfish (*Cyprinodon n. amargosae* Miller) were collected by minnow trap and dip net from the Amargosa River near Dumont Dunes, San Bernardino County, California. Fish were transported to the Center for Aquaculture and Aquatic Biology at the University of California, Davis, and maintained in 1.2 m diameter, flow-through tanks at 26-28°C under ambient photoperiod. All procedures were approved by the Animal Care and Use Committee of the University of California, Davis.

Pupfish breeding

On 20 December 2003, wild-caught pupfish were spawned in two groups of six females and three males in 114-liter tanks. These spawning tanks were maintained at 29.10±0.30°C (mean ± s.e.m.) and 0.4 p.p.t. salinity. The bottom of each tank was covered with cheesecloth. Pupfish spawned their eggs onto the cheesecloth, which was then removed from the spawning tank and placed into aerated 2-liter beakers. A single drop of acriflavine (Novalek, Inc., Hayward, CA, USA) was added to each beaker to prevent fungal growth. Eggs were maintained at 31.28±0.52°C until hatching (up to 10 days).

On the day of hatching, larval pupfish were transferred to 2-liter, aerated buckets (32.06±0.75°C; 0.4 p.p.t.) and fed a diet of Liquifry No. 1 (Interpet, Ltd, Dorking, Surrey, England) and live brine shrimp nauplii (San Francisco Bay Brand, Inc., CA,

USA). On 4 January 2004, at 15 days post-fertilization (d.p.f.), larval pupfish from both breeding tanks were photographed for later body length measurement and divided evenly into experimental treatments.

Food availability and growth rate effects on morphology

In the first experiment, we examined how variation in growth rate influenced morphological development. At 15 d.p.f., larval pupfish were randomly assigned to three treatments: high food availability (100% daily rations, *ad libitum*), medium food availability (50% daily rations), and low food availability (20% daily rations). Given the small size of pupfish at the start of the experiment, food treatments were determined as a proportion of the highest feed amount. In the highest feeding amount, fish were fed to excess so that some food was left on the bottom of the rearing bucket. All treatments were fed brine shrimp and spirulina flake foods (Aquatic Eco-Systems, Inc., Apopka, FL, USA), and maintained at 0.4 p.p.t. salinity. Each ration treatment was replicated (N=8 for high and medium ration treatments; N=7 for low ration treatment due to mortality) in 2-liter buckets with four pupfish per bucket. Morphological values reported represent the mean per bucket. Buckets were maintained in four tanks (approximately 114-liter; 90 cm long×45 cm wide×30 cm high) that were filled ~¼ deep with water. Two buckets from each ration treatment were placed in each tank, to insure that treatments were balanced among tanks. Water temperature was maintained at 33.33±0.09°C by electric heaters. However, minor variation in temperature in the room resulted in slight temperature variation among the four tanks (range, 32.35–34.06°C), so temperature was added *post hoc* to our analyses.

During the experiment, we measured the standard length (*SL*) and total length (*TL*) of pupfish every 21 days beginning at 15 d.p.f. From 15 dpf through 51 d.p.f., length was measured from digital photographs taken with an AxioVision camera connected to a Zeiss Stemi SV11 dissecting microscope and illuminated with a Zeiss KL1500 light (Zeiss, Oberkochen, Germany). Length measurements were later calculated from the digital photos using ImageJ software (Version 1.24, NIH). From 73 d.p.f. through 141 d.p.f., length was measured using calipers (precision ±0.05 mm). At 141 d.p.f., fish were sacrificed (MS222; 250 mg l^{-1} H_2O), and the right side of the body of each fish was photographed using an AxioVision camera attached to a dissecting microscope. At this time, we visually assessed the presence or absence of the paired pelvic fins and photographed the pelvic region of each fish's body for documentation. Digital photographs were then used to quantify body size (*SL*), relative head size (head length measured from tip of the retracted premaxillaries to the posterior edge of opercle/*SL*), relative eye size (diameter of eye/*SL*), and relative body depth (depth of body at posterior end of opercle/*SL*) using ImageJ Software. Morphological data is presented as ratios normalized to body length in order to make comparisons with published descriptions of the morphology of *C. diabolis* in Devil's Hole and the refuges (Wales, 1930; Miller, 1948; Williams, 1977).

Whole-body T_4 radioimmunoassay

To determine whole-body concentrations of the thyroid hormone thyroxine (T_4), each fish was homogenized (polytron PT Kinematica GmbH, Kriens-Luzern, Switzerland) with 1.2 ml ice-cold 100% ethanol containing 1 mmol l^{-1} 5-propyl-2-thiouracil (ETOH-PTU). To determine extraction efficiency, 50 μl (~22 000 c.p.m.) of I^{125}-labeled tri-iodothyronine (T_3) (Perkin-Elmer, Wellesley, MA, USA) were added to each homogenate, and the proportion of I^{125}-T_3 extracted was later measured in duplicate at the time of T_4 radioimmunoassay. The homogenate was sonicated (Sonifier 450, Branson, Danbury, CT, USA) and centrifuged (1409 ***g***) for 20 min at 4°C. The supernatant was then removed and saved for T_4 measurement. The tissue pellet was then resuspended in 0.3 ml ETOH-PTU, centrifuged again, and supernatant was removed and combined with supernatant from the first centrifugation. The supernatant was dried under nitrogen gas, and then resuspended in 100 μl of ice-cold sodium barbital buffer (pH 8.6) containing 0.5% bovine γ-globulin (Sigma) and 1 mmol l^{-1} PTU.

T_4 was measured by radioimmunoassay as described elsewhere (Dickhoff et al., 1982). Samples (10 μl) of extract were incubated for 2 h at 37°C in sodium barbital buffer with anti-$_L$-T_4 antiserum (1:5000; Accurate Chemical & Scientific Corp., Westbury, NY, USA) and I^{125}-labeled T_4 (Perkin-Elmer). Sodium barbital buffer containing 20% polyethylene glycol was then added to each sample, and samples were centrifuged (1409 ***g***) for 20 min at 4°C. The supernatant was removed to separate free and bound hormone, and the remaining pellet was assayed for radioactivity (Cobra II gamma counter, Packard, Downer's Grove, IL, USA). T_4 standards from 1.25 to 60 ng ml^{-1} defined the sensitivity of the assay. All samples were run in duplicate, and the intra-assay coefficient of variation was 13.4%.

Extraction efficiency (mean ± s.e.m.) of I^{125}-T_3 from pupfish bodies was 61.4±4.3%. We found that extraction efficiency was negatively associated with body mass [extraction efficiency=0.922–1.798(body mass); r^2=0.86, $F_{1,21}$=131.9538, P<0.0001], and since mean body size differed among food ration treatments (Fig. 1), extraction efficiencies varied among treatments (ANOVA, $F_{2,20}$=41.3110, P<0.0001). Given these effects of body size on extraction efficiency, we corrected measurements of whole-body T_4 levels by the extraction efficiency for each fish.

Inhibition of thyroid hormone production

In the second experiment, we examined how the pharmacological inhibition of thyroid hormone production affected morphological development. At 15 d.p.f., larval Amargosa River pupfish were assigned to 2-liter buckets, at a density of four pupfish per bucket. These buckets were then divided into three treatments. One treatment was administered 0.03 mmol l^{-1} methimazole (Sigma), a second group received 0.01% $KClO_4$ (potassium perchlorate; Sigma), and the third group served as a control. Methimazole and $KClO_4$ are established pharmacological inhibitors of thyroid hormone production from the thyroid gland (Wolff, 1998; Roy et al.,

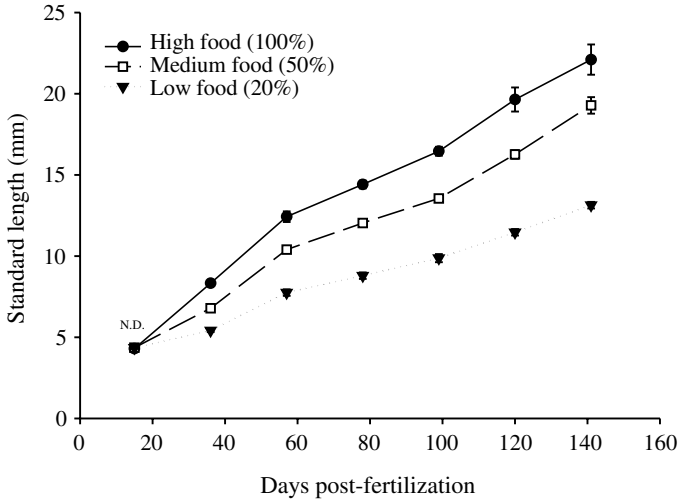

Fig. 1. The growth rate of pupfish from the high food (100% rations), medium food (50% rations), and low food (20% rations) treatments differed (P<0.0001). Tukey pairwise comparisons showed no difference (ND) in body length among the three treatments on the 1st measurement day (15 days post-fertilization) when fish were initially assigned to treatments. Body size deviated significantly by day 36 and remained different among treatments through the conclusion of the experiment on day 141. Values are means ± s.e.m. (N=7–8).

2004). Pupfish were treated with methimazole and $KClO_4$ by dissolving these compounds in water and adding them to the rearing buckets. Water in all treatment buckets was changed every other day. All treatments (N=8 for control and $KClO_4$ treatments, N=7 for methimazole treatment) were fed the 100% daily rations of brine shrimp and spirulina flake foods as described above, and maintained at 33.04±0.19°C (mean ± s.e.m.; range, 30.59–34.75°C) and 0.4 p.p.t. At the beginning (15 d.p.f.) and end (68 d.p.f.) of the experiment, all fish were photographed digitally with an AxioVision camera connected to a Zeiss Stemi SV11 dissecting microscope, and morphological measurements were later made from these images using ImageJ software.

Statistical analyses

We used a repeated-measures ANOVA model to examine the effects of treatment and measurement day on the standard length of pupfish in the low, medium and high food treatments. We then used Tukey HSD tests (overall α=0.05) to calculate multiple paired comparisons among the three growth treatments for each measurement day. Given that morphological values were percentages, we arcsine transformed morphological data from the food ration and goitrogen experiments prior to analysis. A two-factor ANOVA model was used to determine whether there were effects of feeding amount and rearing temperature on head size, eye size, body depth, and on the percentage of fish with pelvic fins. When the model revealed a significant treatment effect, multiple pairwise comparisons among the three treatments were calculated using a Tukey HSD test. We tested for differences in whole-body levels of thyroid hormone (T_4) among growth rate treatments using a one-factor ANOVA model followed by a Tukey HSD multiple pairwise comparison.

For the goitrogen experiment, we first used a one-factor ANOVA model to determine if there were any initial differences in standard length among treatments at the start of the experiment. We also used a two-factor ANOVA to determine whether there were effects of treatment and rearing temperature on standard length when the experiment was terminated. We then used a Tukey HSD test for pairwise comparisons among treatments. Two-factor ANOVAs were used to examine how treatment and temperature affected head size, eye size, body depth and pelvic fin development. Multiple pairwise comparisons among treatments were then calculated using Tukey HSD tests. All statistical tests were two-tailed and performed using JMP 4.0.2 software (SAS Institute, Inc.).

We present and analyze morphological values as ratios normalized to body length to compare with the original description of *C. diabolis* and background literature on morphology of this species, which describes the morphology as normalized ratios (Wales, 1930; Miller, 1948; Williams, 1977). Such normalized ratios, however, can introduce biases that confound statistical analyses and result in erroneous conclusions (Packard and Boardman, 1999). For the food ration and goitrogen experiments, we therefore performed a second set of analyses on absolute measurements of head size, eye size and body depth using ANCOVA models with treatment, water temperature, body length and their interactions as factors. Only the statistically significant interactions of these models are presented. These secondary analyses provided an important confirmation of the conclusions drawn from statistical analysis on the ratio values.

Results

Food ration and growth rate affect morphological development

Food ration levels generated significant differences in growth rates (Fig. 1; repeated-measures ANOVA, treatment effect, $F_{2,17}$=157.663, P=<0.0001; water temperature effect, $F_{1,17}$=1.055, P=0.319; treatment×temperature interaction, $F_{2,17}$=0.007, P=0.9932; time effect, $F_{6,12}$=9.021, P=0.0007). Pairwise comparisons among treatments on each sampling day showed no difference among treatments on the day when fish were initially assigned to treatments (15 d.p.f.). However, body sized differed on all subsequent sampling days.

Fish in the low ration treatment exhibited morphological characteristics similar to *C. diabolis* in Devil's Hole. Pupfish reared in the low food treatment showed a proportionally larger head size ($F_{2,17}$=3.764, P=0.0443), larger eye diameter ($F_{2,17}$=5.822, P=0.0119), and shallower body depth ($F_{2,17}$=23.665, P<0.0001) than fish in the high and medium food treatments (Fig. 2). We found no effect of water temperature on head size ($F_{2,17}$=2.255, P=0.1515), eye size

($F_{2,17}$=1.822, P=0.1948) or body depth ($F_{2,17}$=0.121, P=0.7320). Significantly fewer fish in the low food treatment (14%) developed pelvic fins compared to fish in the medium food (66%) and high food treatments (78%) (Fig. 3A; $F_{2,17}$=7.446, P=0.0048). Fish from all three treatments developed pelvic fins less often at warmer rearing temperatures (Fig. 3B; $F_{1,17}$=4.990, P=0.0392), and there was no interaction between treatment and rearing temperature ($F_{2,17}$=0.655, P=0.5319).

The secondary analysis of the morphological results using ANCOVA models similarly showed that food ration treatment affected head size (r^2=0.9867, treatment, $F_{2,11}$=4.0055, P=0.0493; body length, $F_{1,11}$=33.2845, P=0.0001), eye size (r^2=0.9770, treatment, $F_{2,11}$=7.1028, P=0.0105; body length, $F_{1,11}$=7.0300, P=0.0225), and body depth (r^2=0.9929, water temperature, $F_{1,11}$=8.1175, P=0.0158; body length, $F_{1,11}$=3.1236, P=0.0001; water temperature×body length interaction, $F_{1,11}$=5.0161, P=0.0468; treatment×water temperature×body length interaction, $F_{2,11}$=4.4102, P=0.0392).

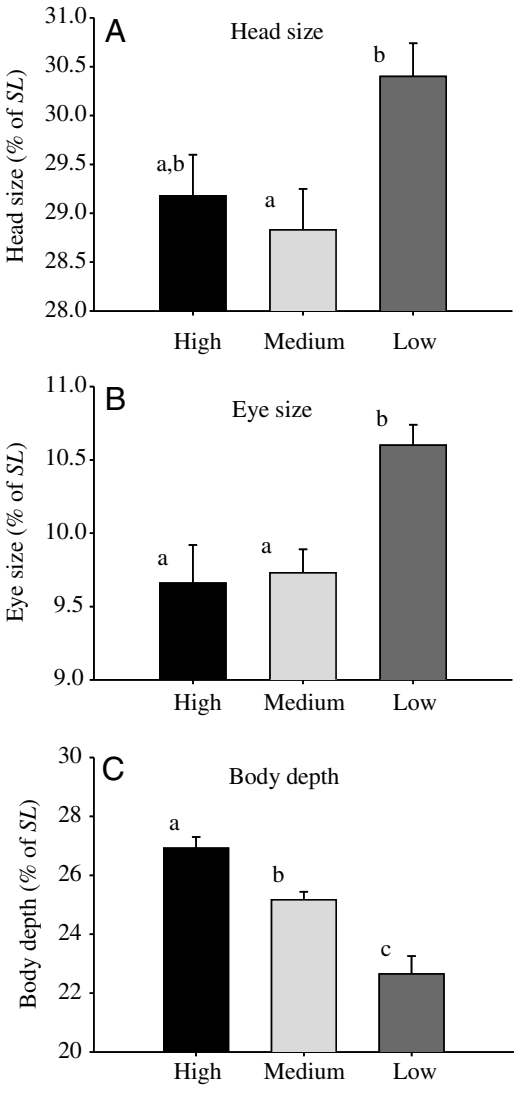

Fig. 2. Relative (A) head size (P=0.0443), (B) eye size (P=0.0119) and (C) body depth (P<0.0001) differed in pupfish from high food (100% rations), medium food (50% rations), and low food (20% rations) treatments. Values are means ± s.e.m. (N=7–8). Letters indicate pairwise comparisons among groups (Tukey HSD test). *SL*, standard length.

Food ration effects on whole-body T_4

The amount of T_4 per fish differed among food ration treatments (Fig. 4; $F_{2,20}$=43.8699, P<0.0001). The low ration treatment had a mean (± s.e.m.) whole-body T_4 level of 0.22±0.03 ng/fish, the medium ration treatment had 1.07±0.07 ng/fish, and the high ration treatment had 0.77±0.08 ng/fish. The amount of T_4 in whole-body tissues was also positively associated with body mass ($F_{1,21}$=9.5307, P=0.0056), which confounds interpretation of treatment differences in whole-body T_4 since body size also differed among treatments (Fig. 1).

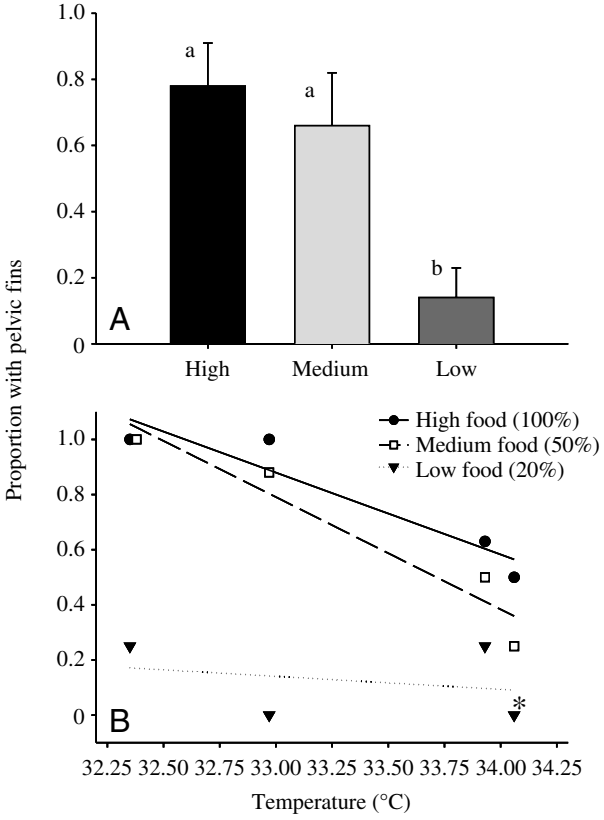

Fig. 3. Effect of food ration on pelvic fin development. (A) A significantly smaller proportion of fish in the low food ration treatment developed pelvic fins when compared to the medium and high food treatments (P=0.0048). Values are means ± s.e.m. (N=7–8), and letters indicate pairwise differences (Tukey HSD test). (B) In all treatments, there was a trend toward a low proportion of fish with pelvic fins when reared at higher temperatures (P=0.0392). Each data point represents the mean of two replicated buckets per temperature value (except the point indicated by asterisk, which represents a single bucket).

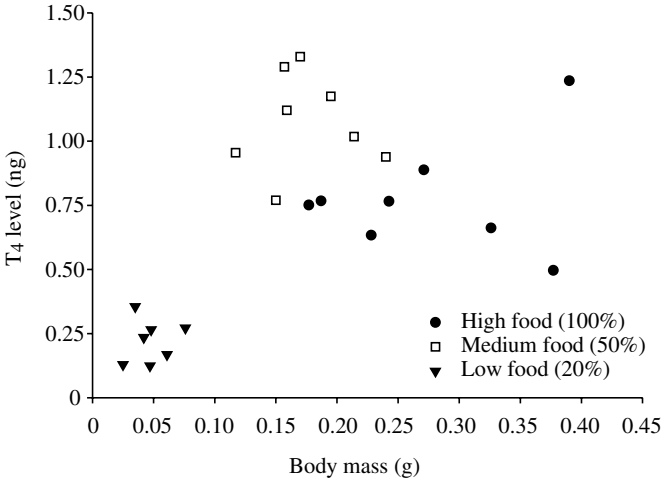

Fig. 4. Food ration treatments differed in whole-body levels of the thyroid hormone T_4 (P<0.0001). T_4 levels are plotted against body mass (g) for each treatment bucket.

Goitrogens alter morphology

Fish that received known goitrogens (inhibitors of thyroid hormone production) developed larger heads (Fig. 5; $F_{2,17}$=7.404, P=0.0053) and eyes ($F_{2,17}$=10.353, P=0.0013), but a smaller body depth ($F_{2,17}$=7.542, P=0.0049) compared to control fish. Temperature did not affect relative head growth (temperature effect, $F_{1,17}$=0.724, P=0.4075; temperature×treatment interaction, $F_{2,17}$=0.099, P=0.9056). Elevated rearing temperature did, however, generate fish with a larger relative eye size (Fig. 6A; temperature effect, $F_{1,17}$=11.739, P=0.0035; temperature×treatment interaction, $F_{2,17}$=1.090, P=0.3600) and smaller relative body depth (Fig. 6B; temperature effect, $F_{1,17}$=6.419, P=0.0221; temperature×treatment interaction, $F_{2,17}$=0.329, P=0.7246). A second analysis of morphological data using ANCOVA models likewise showed that treatment with goitrogens influenced head size (r^2=0.9598, treatment, $F_{2,11}$=5.4024, P=0.0232; body length, $F_{1,11}$=27.0258, P=0.0003) and body depth (r^2=0.9578, treatment, $F_{2,11}$=5.9448, P=0.0178; body length, $F_{1,11}$=13.1662, P=0.0040; water temperature×body length interaction, $F_{1,11}$=5.4475, P=0.0396). There was not, however, an effect of goitrogen treatment on eye size when the data was analyzed using an ANCOVA model (r^2=0.6664, treatment, $F_{2,11}$=3.1809, P=0.0813).

Pupfish in goitrogen treatments showed a reduction in pelvic fin development when compared to control fish (Fig. 7A; $F_{2,17}$=10.863, P=0.0009). We also found that higher water temperatures inhibited fin development (Fig. 7B; $F_{1,17}$=6.640, P=0.0196). There was no significant interaction between treatment and temperature ($F_{2,17}$=0.242, P=0.7874).

Overall, these shifts in body proportions did not appear to be caused by a general inhibition of growth. Mean body length of pupfish among treatments was similar at the beginning of the experiment ($F_{2,20}$=2.843, P=0.0819). At the end of the experiment, treatments still did not differ in body length ($F_{2,17}$=1.815, P=0.1930), indicating that goitrogens did not alter

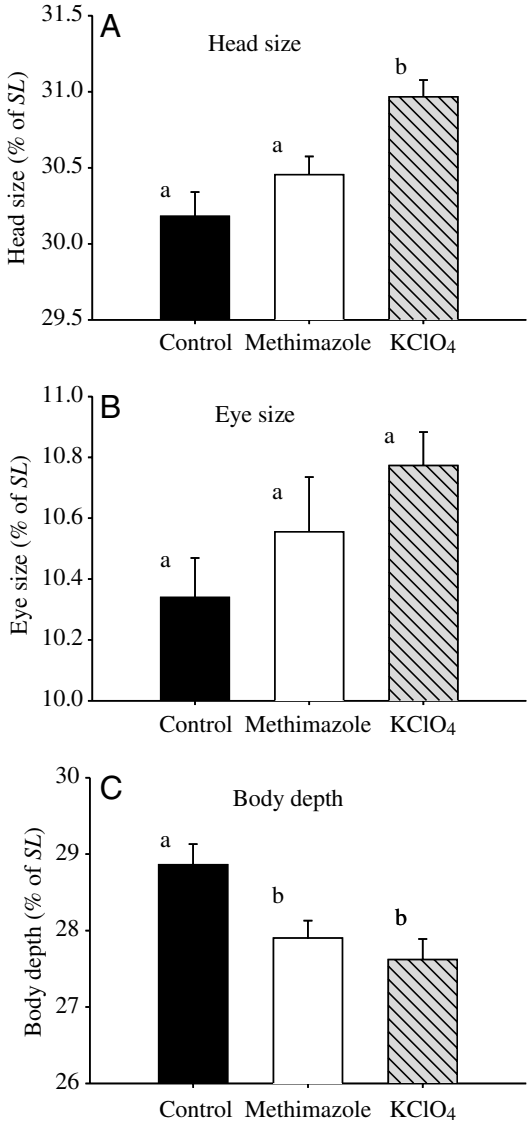

Fig. 5. Effect of goitrogen treatment on pupfish morphology. Relative (A) head size (P=0.0053), (B) eye size (P=0.0013) and (C) body depth (P=0.0049) of pupfish varied significantly among the methimazole, $KClO_4$, and control treatments. Relative head size, eye size, and body depth are presented as percent of standard length (*SL*). Values are means ± s.e.m. (N=7–8). Letters indicate pairwise comparisons among groups (Tukey HSD test).

growth rate. Elevated water temperature, however, resulted in a reduction in body size in all treatments ($F_{1,17}$=41.630, P<0.0001). There was no interaction effect of treatment and temperature on body size ($F_{2,17}$=0.018, P=0.9825).

Discussion

In this study, we tested whether simple manipulations of growth rate *via* diet could influence morphological development in Amargosa River pupfish. Our results show that low food availability can developmentally generate a dwarfed morphological phenotype that resembles that of *C. diabolis* in

Devil's Hole. Pupfish reared under low food ration developed larger head and eye sizes, shallower bodies, and failed to develop paired pelvic fins. *Post hoc* analysis of variation in rearing temperature revealed that elevated temperature also reduced the percentage of individuals developing pelvic fins. This effect was apparent even though rearing tanks only differed slightly in water temperature (<2°C), and suggests that even small temperature differences can generate significant morphological variation in developing pupfish.

We also found that low food ration was associated with a reduction in whole-body levels of the thyroid hormone T_4, suggesting that morphological changes associated with low food ration may have occurred in part *via* changes in thyroid hormone homeostasis. Supporting this idea, larval pupfish given goitrogens to block endogenous thyroid hormone production developed larger heads and eyes and shallower bodies. Also, fewer fish in the goitrogen treatments developed pelvic fins. Further analysis revealed that elevated rearing temperature caused pupfish to develop larger eyes and shallower body depth. Taken together, these results indicate that elevated temperatures and reduced food intake can developmentally generate a morphological phenotype similar to that expressed by *C. diabolis* in Devil's Hole, and that these morphological changes may occur in part through changes to thyroid hormone physiology.

The morphological plasticity in pupfish seen here strongly suggests that habitat differences in food availability and temperature contribute to the morphological differences seen between *C. diabolis* in the refuges and Devil's Hole. This plasticity may mediate the increase in body size, reduction in head and eye size, and increase in body depth seen in all refuge populations of this species. One of the primary concerns in *C. diabolis* management has been whether pupfish from the refuges could be successfully reintroduced into Devil's Hole. The altered morphology of refuge fish might preclude their survival upon release into the energetically challenging (high temperature, low food) environment of Devil's Hole. However, if the morphological deviations of refuge populations are due

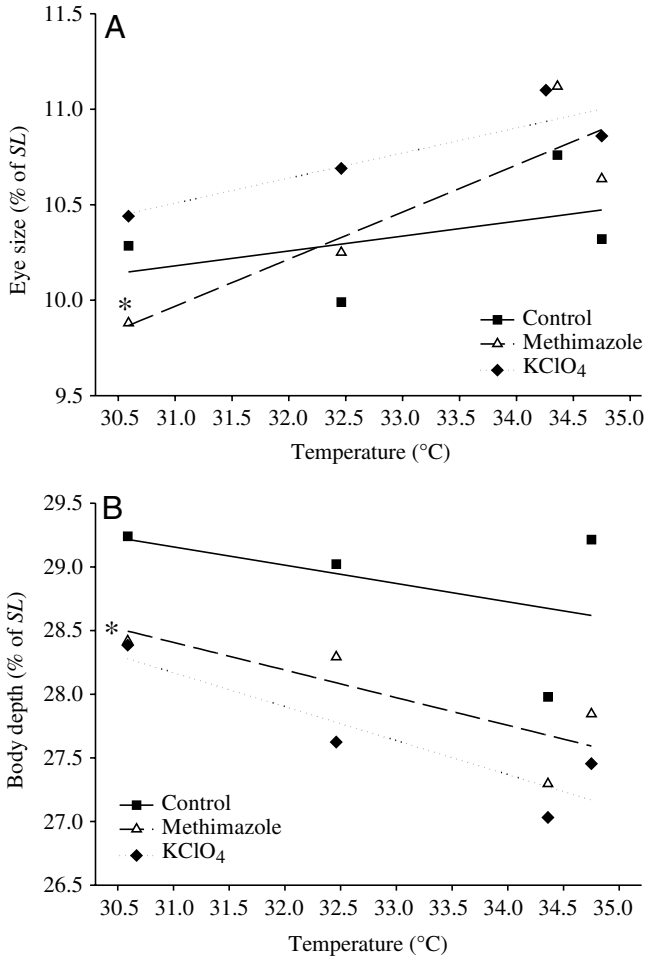

Fig. 6. Elevated rearing temperature increased (A) relative eye size (P=0.0035) and decreased (B) relative body depth (P=0.0221) in the methimazole, $KClO_4$ and control treatments. Each data point represents the mean of two replicated buckets per temperature value (except the point indicated by asterisk, which represents a single bucket).

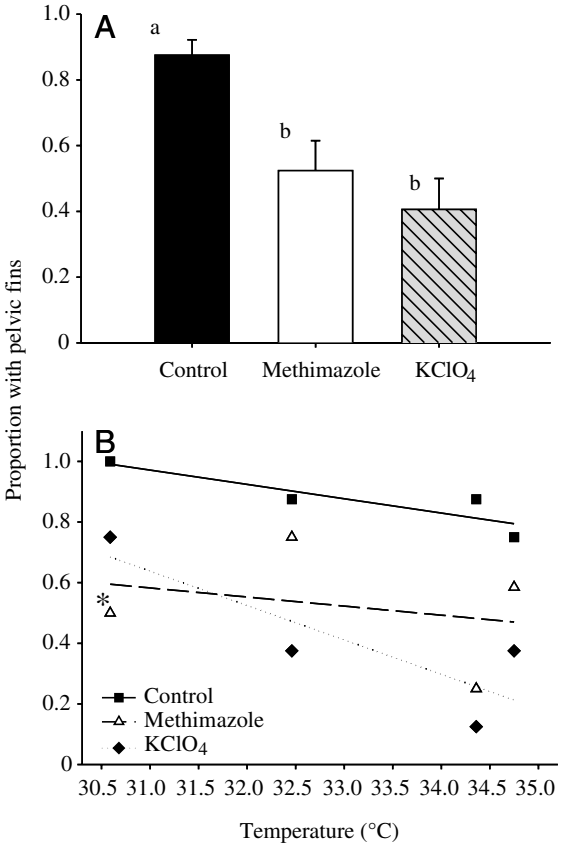

Fig. 7. Effect of goitrogen treatment on pelvic fin development. (A) A significantly smaller proportion of fish in the methimazole and $KClO_4$ treatments developed pelvic fins when compared to the control (P=0.0009). Values are means ± s.e.m. (N=7–8) and letters indicate pairwise differences (Tukey HSD test). (B) The proportion of fish with pelvic fins plotted against rearing temperature for each treatment. Elevated rearing temperatures resulted in a lower proportion of fish developing pelvic fins (P=0.0196). Each data point represents the mean of two replicated buckets per temperature value (except the point indicated by asterisk, which represents a single bucket).

to developmental plasticity, as suggested here, then these deviations might be reversed by altering rearing conditions. Eggs or newly hatched larvae transferred from the refuges to Devil's Hole might still develop the Devil's Hole morphology. This prediction could be tested by rearing refuge larvae under conditions that more closely resemble those of Devil's Hole.

It is important to note that, although environmental conditions generated a morphology similar to that seen in Devil's Hole, we did not recreate the natural phenotype of *C. diabolis* in Devil's Hole. For instance, relative head length was quantified as 33.5–37.5% (mean, 35.4%) of standard length in *C. diabolis* from Devil's Hole (Miller, 1948). Yet, head length only increased from 29.0% (high food ration) to 30.5% (low food ration) in the experimental *C. n. amargosae* fish shown here. Environmental conditions can thus developmentally shift Amargosa River pupfish toward a Devil's Hole morphology, but do not seem to have replicated that phenotype. Even in the refuge populations, Williams found a reduction of relative head length in *C. diabolis* to ~32% (means for males, 31.6%; females, 33.4%) (Williams, 1977), indicating that the morphology of fish in the refuges is closer to fish in Devil's Hole than are the experimental Amargosa River pupfish presented here.

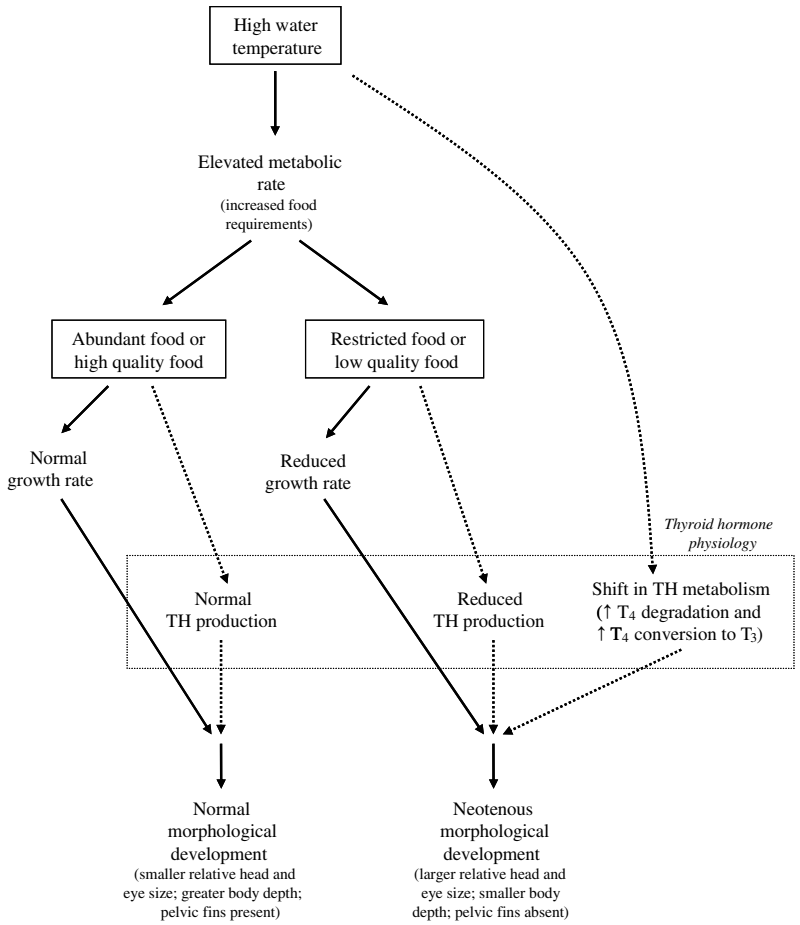

Fig. 8. Morphological plasticity in pupfish may be driven by differences in environmental conditions that affect thyroid hormone physiology and, subsequently, morphological development. This model illustrates a possible scenario where high water temperatures elevate metabolism and cause a suite of physiological changes in the thyroid hormone system that are dependent on food availability and quality. If food resources are abundant or high in nutritional quality, pupfish develop a normal morphology with small head and eye sizes, a deep body, and pelvic fins (possibly occurring in the refuges for *C. diabolis*). If food resources are scarce or of low quality, however, thyroid hormone production may be inhibited resulting in development of the neotenous morphology (large head and eye sizes, small body depth, lack of pelvic fins) typical of *C. diabolis* in Devil's Hole.

Ecophysiology of morphological change

Based on our results, we propose a model for how environmental differences may have caused morphological shifts among populations of *C. diabolis* (illustrated in Fig. 8). Stepping through this figure, our model suggests that the elevated water temperature of Devil's Hole combined with low availability or quality of food resources leads to a reduction in growth rate and depression of thyroid hormone status in juvenile *C. diabolis*. This combination of low growth and reduced thyroid hormone levels during the developmental transition from larval to juvenile morphologies generates the neotenous or 'dwarfed' morphology typical of *C. diabolis* in its native habitat. Applying this model to our study system, the refuge habitats are generally lower in temperature and higher in food resources (i.e. algae, invertebrates) than Devil's Hole (Wilcox, 2001; Karam, 2005). Thus, for *C. diabolis* in the refuges, the lower temperature combined with the greater abundance of food facilitates the development of a morphology typical for other pupfish species. This morphology (larger and deeper body, smaller head and eyes) is a deviation from the Devil's Hole phenotype.

To understand this model, we must clarify the links between water temperature, food availability and thyroid hormone physiology. First, with respect to temperature, in desert pupfishes as in other fishes, it is well established that elevated water temperatures result in increased metabolic demands (Brett and Groves, 1979; Peck et al., 2005). For example, Salt Creek pupfish (*C. salinus salinus*) from the Death Valley clade have higher metabolic rates, with higher temperatures in both freshwater and ½ seawater (Stuenkel and Hillyard, 1981). What is more, desert pupfish (*C. macularius*) acclimated to higher temperatures ingest more food than those acclimated to lower temperatures due to an elevated metabolic rate and a depressed assimilative efficiency for food (Kinne, 1960). It follows that *C. diabolis* occupying the high thermal environment of Devil's Hole (~33–34°C) likely have elevated food and oxygen consumption rates to meet the increased cellular nutrient demands of this elevated temperature environment.

As temperatures become elevated, metabolic demands

increase. If food resources in a high temperature habitat are abundant or of high quality, Devils Hole pupfish can acquire sufficient nutrients to maintain their elevated metabolism and still grow rapidly. However, in habitats where food resources are of low quality or where competition for limited resources is high, pupfish may have difficulty obtaining enough food to meet elevated metabolic demands while providing for growth and reproduction. Some of the refuges have standing algal crops that are considerably higher than Devil's Hole (Karam, 2005), which may be related to differences in solar insolation between habitats. Devil's Hole receives direct sunlight for only a few hours each day during summer, while the refuges receive year-round sun exposure. The species composition of food resources also differs between the refuges and Devil's Hole (Williams, 1977; Karam, 2005). This variation in food resources may generate population differences in energy allocation and growth for *C. diabolis*.

Next, temperature and food resources alter the production of thyroid hormones (Leiner and MacKenzie, 2003; MacKenzie et al., 1998). In trout, elevated temperature increases the degradation rate of the thyroid hormone T_4 as well as the rate of deiodinase conversion of T_4 to triiodothyronine (T_3) (Eales et al., 1982; Johnston and Eales, 1995). Similarly, restricted food intake can reduce plasma levels of T_4 and T_3 in fish (Eales and Shostak, 1985; Eales, 1988; Power et al., 2000; Reddy and Leatherland, 2003). Even food of low nutrient quality can reduce growth rates and thyroid hormone production (Higgs and Eales, 1979; Eales et al., 1993; MacKenzie et al., 1993). Yet, what is not commonly appreciated in fish is that this variation in thyroid physiology during early life may have a dramatic impact on morphological development.

Thyroid hormones play a key role in mediating the larval to juvenile transition in fishes (Inui and Miwa, 1985; Brown, 1997; Trijuno et al., 2002). We propose that variation in thyroid hormone physiology induced by temperature and food conditions during this transition may generate morphological differences among *C. diabolis* in Devil's Hole and the refuges. For example, zebrafish (*Danio rerio*) larvae treated with goitrogens have smaller bodies, larger heads, and fail to develop pelvic fins (Brown, 1997), but the addition of exogenous thyroid hormone to the water prevents these changes. Goldfish (*Carassius auratus*) larvae that receive supplemental thyroid hormone accelerate their growth and show an earlier differentiation of fin development (Reddy and Lam, 1992). Changes to thyroid hormone physiology are also thought to mediate the neotenic morphology that typifies some species. Adult ice gobies (*Leucopsarion petersii*) display several morphological features, such as the absence of scales and presence of reduced pelvic fins – characteristics that typify juveniles in other gobies. The appearance of a thyroid gland in the ice goby is also developmentally delayed, suggesting that thyroid physiology, in part, mediates the neotenous morphology of this species (Harada et al., 2003a; Harada et al., 2003b).

Additional work is needed to test the model that we have proposed here. Specifically, quantification of metabolic rates, growth trajectories, and food consumption parameters for *C. diabolis* in Devil's Hole and the refuges is needed. As the model is currently described, we address only two environmental factors, temperature and food, which may generate the morphological changes seen in refuge populations of *C. diabolis*. Yet, other environmental factors could play a role in generating these morphological shifts. For instance, mean dissolved oxygen saturation is lower in Devil's Hole (1–2 mg l^{-1}) than in the refuges (4–5 mg l^{-1}) (Wilcox, 2001; Karam, 2005). Studies in other fishes have shown that low dissolved oxygen can reduce serum levels of thyroid hormone (Wu et al., 2003). It is also possible that Devil's Hole may have distinct water chemistry characteristics that contribute to the dwarfed morphology of pupfish in the habitat. While the water analyses that have been conducted for Devil's Hole do not present any obvious chemical candidate that would cause pupfish in this habitat to show a unique morphology compared to the other habitats occupied by pupfish in the Death Valley region (i.e. Walker and Eakin, 1963; Dudley, Jr and Larson, 1974), there are a number of chemicals not measured in these analyses that are known to cause defects in thyroid hormone production in vertebrates. For example, iodine, selenium and lithium can all impact growth and development by altering thyroid function, and future analyses should quantify these compounds in Devil's Hole water. Lastly, while our current model focuses on how food resources change thyroid hormone physiology, other metabolic hormones could also alter morphological development. Nevertheless, the utility of the model we propose is that it provides testable predictions for the cause of morphological shifts among *C. diabolis* populations and potentially other endangered fish species as well.

Implications of phenotypic plasticity for management of imperiled species

Management programs for imperiled species often emphasize genetic health defined as maintaining a genetically effective population size, preventing inbreeding and avoiding artificial selection (Meffe, 1986; Philippart, 1995). Such goals have been key considerations in the management of *C. diabolis* (Baugh and Deacon, 1988; US Fish and Wildlife Service, 1990). It has been estimated that Devils Hole pupfish have been naturally isolated for ~20 000 years (Miller, 1981), and molecular evidence shows that the species is genetically distinct from other pupfishes in the Death Valley clade (Echelle and Dowling, 1992; Duvernell and Turner, 1998; Duvernell and Turner, 1999; Martin and Wilcox, 2004). An initial emphasis on maintaining the genetic integrity of the refuge populations thus was fitting. Yet, the morphological shifts that occurred in these populations illustrate how the developmental effects of altered habitat conditions must also be considered when managing species.

In the case of *C. diabolis*, the population in Devil's Hole has declined over the last few years to a precarious size, and a census of Devil's Hole in April 2006 recorded fewer than 50 individuals. Artificial propagation is currently underway to ensure that the species will not become extinct in the short term. These propagation efforts include both the expansion of *C. diabolis* populations in refuge habitats and a renewed attempt to spawn and rear the species in captivity. Given the morphological

plasticity documented here, it is crucial that environmental conditions in these efforts are strictly managed to mimic the conditions of Devil's Hole and ensure that captively propagated *C. diabolis* develop a morphology typical of the species in its natural habitat. There is warranted concern that *C. diabolis* showing a larger body size and altered morphology might have difficulty obtaining sufficient food for routine body maintenance and reproduction if reintroduced into Devil's Hole. Careful control over environmental conditions during artificial propagation, however, should help avoid those potential pitfalls.

More generally, the consequences of environmentally induced plasticity are only beginning to be recognized in conservation biology even though plastic phenotypic shifts have been recorded in imperiled species for many years. For instance, black-footed ferrets (*Mustela nigripes*) bred and raised in captivity have shorter-length bones in the forearms and rear legs, and these changes appear to result from plastic responses to rearing conditions (Wisely et al., 2005). Indeed many animals reared in captivity show behavioral changes that impede the success of reintroduction and supplementation programs (Snyder et al., 1996; Wallace, 2000). For example, breeding large numbers of fish in hatcheries has long been an approach to supplement and restore wild populations; yet fish reared in hatcheries can show behavioral differences from their wild counterparts, and many of these behavioral changes can be attributed to their rearing environments (Olla et al., 1994; Berejikian et al., 1996; Braithwaite and Salvanes, 2005). Differences in brain size have even been found between fish reared in hatcheries and the wild, and these differences appear to be generated in part through plastic developmental responses to the environment (Marchetti and Nevitt, 2003; Lema et al., 2005; Kihslinger and Nevitt, 2006; Kihslinger et al., 2006). While such plastic changes in brain and behavior are not often taken into account in conservation, neurobehavioral changes in altered environments might be more widespread than commonly considered, given that laboratory studies with mammals and fish provide abundant examples of environmental influences on neural phenotype (i.e. Diamond et al., 1993; van Praag et al., 2000; Lema, 2006).

Nevertheless, our understanding of the physiology and mechanisms of phenotypic development is only beginning to be incorporated into new solutions for conservation problems, and many questions remain to be explored (Carey, 2005). Can habitats be intentionally restored such that they take into account the expression of phenotypes (Watters et al., 2003)? Or, will plasticity bolster the survival of plant and animal species as they face anthropogenic changes in their environment? Answering these questions requires an increased attention to the role of phenotypic plasticity in conservation biology and could generate innovative approaches for protecting imperiled species (e.g. Watters et al., 2003).

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