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FEATURE ARTICLE

Echinoid larvae can express food-conditioned morphological plasticity at ecologically relevant culture densities

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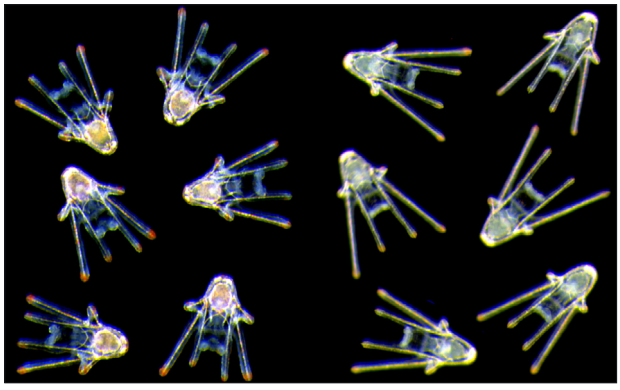
ABSTRACT: The feeding larvae of many echinoids develop long postoral arms relative to body length when food is sparse but relatively short postoral arms when food is abundant, a response thought to adaptively adjust feeding capability. However, in an important recent study, larvae of *Dendraster excentricus* exhibited this food-conditioned plasticity only when reared at a high density typical of laboratory cultures; when reared at a lower density more representative of larval densities in nature, they did not exhibit this plastic response. This finding suggests that laboratory results cannot be easily extended to make inferences about phenotypic plasticity in nature. We replicated this study and extended it to an even lower larval culture density and to a second species, *Lytechinus pictus*. Larvae of *D. excentricus* developed longer arms adjusted for body length when fed the lower of 2 food rations at all culture densities, though differences were only marginally significant at the lower culture density in one experiment. Larvae of *L. pictus* tended to develop longer arms adjusted for body length at lower food rations, though differences only approached statistical significance at the highest culture density in one experiment. For both species, contrasts between food rations almost always showed an inverse relationship between postoral arm length and stomach length, consistent with prior work demonstrating trade-offs in investment in these 2 features characteristic of phenotypic plasticity. These results suggest that the feeding larvae of echinoids may exhibit food-conditioned plasticity of postoral arm length even at low natural densities.



Sibling larvae of the sand dollar *Dendraster excentricus* reared with abundant (left) or scarce (right) food.

Illustration: Peter Nilsson

KEY WORDS: Echinoid · Phenotypic plasticity · Culture density · *Dendraster excentricus* · *Lytechinus pictus*

1. INTRODUCTION

The suspension-feeding larvae of many echinoids exhibit food-conditioned phenotypic plasticity of the structure used for particle capture, the ciliary band, with food-limited larvae developing longer ciliary bands relative to body size than well-fed larvae (reviewed by McAlister & Miner 2018). This plasticity may be adaptive (Strathmann et al. 1992), potentially allowing larvae to compensate for food scarcity by increasing their feeding rate while allowing larvae with ample food to invest in post-larval structures

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rather than in ephemeral feeding structures which are lost at metamorphosis.

However, results of an important recent study complicate the interpretation of laboratory studies of phenotypic plasticity in echinoid larvae. Kacenas & Podolsky (2018) investigated the effect of competition on plasticity in larvae of the sand dollar *Dendraster excentricus*. Larvae reared at culture densities of 0.25 larvae ml^{-1} developed longer postoral arms (a proxy for ciliary band length) adjusted for body length when fed a limited ration than when provided with ample food, consistent with previous experiments (Boidron-Metairon 1988, Hart & Strathmann 1994, Miner 2007, Nguyen et al. 2021). In contrast, adjusted postoral arm length did not vary with food ration when larvae were reared at a culture density of only 0.05 larvae ml^{-1}, a density lower than used in any previous plasticity experiments but comparable to the highest densities of zooplankton found in field surveys (Kacenas & Podolsky 2018). This suggests that the results of laboratory studies on food-conditioned phenotypic plasticity in echinoid larvae, a major focus of larval biology for the past 3 decades, cannot be easily extended to natural settings.

Kacenas & Podolsky's (2018) findings are based on a single experiment which, given its implications, merits replication. In this study, we replicated their experiment and extended it to an even lower culture density as well as to a second echinoid species, the sea urchin *Lytechinus pictus*. We used these experiments to address the question: can larvae of echinoids exhibit food-conditioned phenotypic plasticity when reared at low densities similar to those they likely experience in nature?

2. MATERIALS AND METHODS

2.1. Collection of adults, spawning, and fertilization

Adult echinoids were collected from 2 sites near San Pedro, California: *Dendraster excentricus* from the intertidal zone at Cabrillo Beach (33.709° N, 118.278° W) and *Lytechinus pictus* from the subtidal zone near White Point (33.714° N, 118.317° W). Adults of both species were kept in a recirculating seawater system maintained at 16°C until use. Adults were induced to spawn by injection of ~1 ml (*D. excentricus*) or ~0.4 ml (*L. pictus*) of 0.53 M KCl into the perivisceral coelom (Strathmann 1987). For each of our 4 experiments (2 using *D. excentricus* and 2 using *L. pictus*), eggs of 3 females were kept separate and rinsed with seawater that had been passed through a 0.2 µm filter (filtered seawater, FSW). Eggs of each female were then fertilized with dilute sperm of one of 3 males, yielding 3 separate families with no shared parents. We allowed each family of embryos to develop at 16°C in 500 ml of unstirred FSW for 1 d, then decanted hatched blastulae into a new beaker for each family. The concentration of blastulae in each of these beakers was estimated from Bogorov tray counts of five 0.5 ml samples. Samples of blastulae were killed with dilute formalin prior to counting.

2.2. Algal culture

Rhodomonas lens (CCMP739) was cultured at room temperature in sterilized f/2 medium (Guillard 1975) under natural light. Cells were pelleted in a centrifuge to separate them from the culture medium, then resuspended in FSW. The resuspended algae were then passed through a 20 µm Nitex mesh to break up any cell aggregates. The number of cells of *R. lens* in 15 µl of the resulting suspension was counted using a BD Accuri C6 flow cytometer (BD Biosciences). Cell counts were used to calculate the volumes of *R. lens* suspension to add to culture beakers to yield low or high food ration treatments.

2.3. Experiments with *D. excentricus*

The first experiment (*D. excentricus* Expt 1) replicated that of Kacenas & Podolsky (2018), excluding the treatments that used heterospecifics (i.e. larvae other than those of *D. excentricus*) as competitors. We aliquoted the appropriate volume of blastulae into 1000 ml beakers filled with FSW to prepare cultures at both of the larval densities (0.05 and 0.25 larvae ml^{-1}) used in the original paper, with ⅓ of the blastulae in each beaker coming from each of 3 unique families. Half of the beakers within each density treatment were fed a high food ration (5000 cells ml^{-1} *R. lens*) and the others a low food ration (250 cells ml^{-1}), yielding a total of 4 larval density × food ration treatments, each with 5 replicate beakers. The beakers were then placed in a 16°C environmental chamber and stirred with paddles at 4 strokes min^{-1} (Strathmann 2014).

To assess the accuracy of our manipulation of larval density, 3 additional beakers were prepared at each larval density. Larvae in these count-control beakers were fed the higher food ration and stirred (as above) at room temperature to accelerate their development, increasing their size and opacity and making them easier to count accurately. At 3 d post-fertiliza-

tion (dpf), the larvae in each count-control beaker were concentrated into a small (5–20 ml) volume and killed with ethanol. They were then counted to estimate the actual number of blastulae that had been delivered to the experimental beakers at each density.

Larvae in the experimental beakers were reared until 5 dpf, a length of time chosen to approximate the degree of development that larvae attained by 8 dpf at the colder temperatures (10–14°C from fertilization to 3 dpf and 12–14 °C from 3–8 dpf) used by Kacenas & Podolsky (2018). At 3 and 4 dpf, full water changes were performed by forward filtration (Hodin et al. 2019). After each water change, larvae were fed following the same procedure used during larval culture initiation. No water change or feeding was done at 2 dpf, as larvae do not begin feeding until approximately 40 h post-fertilization at 16°C (authors' unpubl. data) and thus do not deplete their food supply during most of this time.

In a second experiment (*D. excentricus* Expt 2), we added another treatment to determine if larvae at an even lower culture density than used by Kacenas & Podolsky (2018) would show a phenotypically plastic response to the abundance of food. We used the same methods as in *D. excentricus* Expt 1 but added a third treatment of only 0.015 larvae ml^{-1}, half of which were fed the higher food ration and half the lower food ration. Like the other 2 density treatments, this treatment included 5 replicate beakers for each food ration. Rather than aliquoting larvae into these beakers, we transferred exactly 5 larvae from each of the 3 families by pipette for a total of 15 larvae $beaker^{-1}$; therefore, no additional count-control beakers were needed for this larval density.

2.4. Experiments with *Lytechinus pictus*

In a third experiment (*L. pictus* Expt 1), we repeated the procedure used in *D. excentricus* Expt 2 with larvae of *L. pictus*, keeping to the same schedule. Finally, in a fourth experiment (*L. pictus* Expt 2), we repeated the methods of *L. pictus* Expt 1 but altered the schedule slightly, taking photos at 7 dpf to allow larvae to develop further, as this species develops more slowly than *D. excentricus.*

2.5. Morphological measurements

At 5 dpf (*D. excentricus* Expts 1 and 2 and *L. pictus* Expt 1) or 7 dpf (*L. pictus* Expt 2), larvae were concentrated on a Nitex mesh submerged in seawater. A small number of larvae were transferred to a slide and relaxed with 7.5% $MgCl_2$ before being killed with dilute formalin. Larvae were oriented dorsal side up to keep the postoral arms in a single plane of focus at the bottom of the slide, then a coverslip elevated with clay feet added. The first 5 larvae encountered per slide (or all larvae, if fewer than 5) were photographed with a QIClick camera (Teledyne Photometrics) mounted on an Olympus BX-51 compound microscope (Olympus Scientific Solutions) using a 10× (*D. excentricus*) or 20× (*L. pictus*) objective. A stage micrometer was also photographed to calibrate measurements. The stomach length (SL), body length (BL), and right postoral rod length (PORL) of each larva (Fig. 1) were measured with the FIJI distribution of ImageJ (Schindelin et al. 2012). The right postoral rod was used as its size is less likely to be influenced by rudiment development than that of the left postoral rod (Hodin et al. 2016).

There were always at least 5 larvae $slide^{-1}$ in all beakers in all experiments except for the 0.015 larvae ml^{-1} density treatment in *L. pictus* Expts 1 and 2, where there were often fewer than 5 larvae remaining

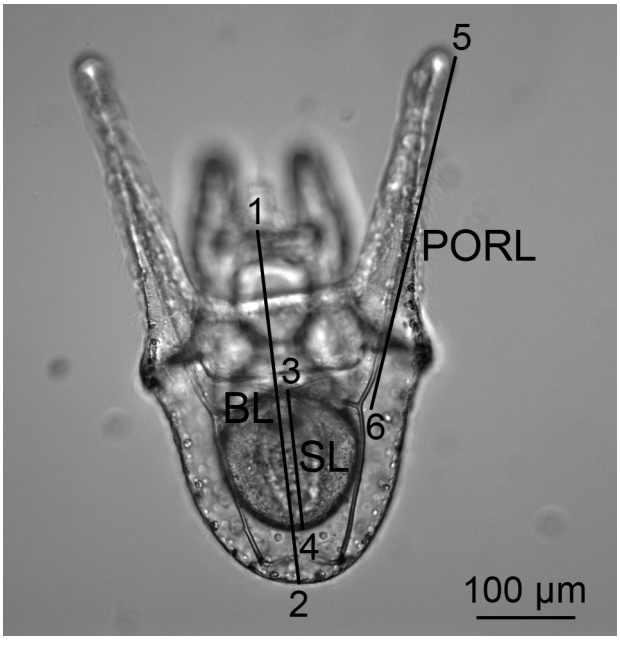

Fig. 1. Landmarks used to derive morphometrics, shown on a larva of *Lytechinus pictus*. All larvae were oriented in dorsal view. BL: body length, measured along the body midline from the edge of the oral hood (1) to the most posterior point on the body (2); SL: stomach length, measured from the most anterior (3) to the most posterior (4) end of the stomach; PORL: right postoral rod length, measured from most anterior point on postoral arm (5) to the junction with the transverse rod (6)

that could be analyzed due to a combination of low survival and high incidence of larvae with missing or obviously broken right postoral rods. Of the ten 0.015 larvae ml^{-1} beakers in *L. pictus* Expt 1, 3 beakers yielded 5 larvae, 6 yielded only 4 larvae, and one yielded only 2 larvae. For *L. pictus* Expt 2, which extended culture duration from 5 to 7 dpf, 5 beakers yielded 5 larvae, one yielded only 4 larvae, one yielded only 2 larvae, 3 yielded only a single larva, and one yielded no larvae. Low survival and abnormal arm morphology past 5 dpf was also noted in a study on culture methods for larvae of the congener *L. variegatus* (Buitrago et al. 2005). While the causes of low survival and abnormal arm morphology in our cultures are unknown, our observations suggest that larvae of *L. pictus* larvae, like those of *L. variegatus* (Lowe & Wray 2000), are particularly fragile and thus more easily damaged during water changes than those of *D. excentricus*.

2.6. Analysis

Studies on feeding structure plasticity in echinoids use diverse statistical approaches in their analyses (McAlister & Miner 2018), a fact that exacerbates the difficulty of comparing results among studies which are already diverse in experimental technique. To enable straightforward comparison with the results of Kacenas & Podolsky (2018), we used the same statistical approach that they did, creating linear mixed-effects models for both dependent variables (PORL and SL) for each experiment. Kacenas & Podolsky (2018) did this using SPSS v.24 (IBM 2016), but we did our analyses in R v.4.1.0 (R Core Team 2021) using the 'lmer' function provided by the 'lme4' v.1.1-27 package (Bates et al. 2015) and extended by the 'lmerTest' v.3.1-3 package (Kuznetsova et al. 2017). Food ration and larval density were treated as fixed effects, beaker as a random effect, BL as a covariate, and either PORL or SL as response variables. Estimated marginal means were calculated in R using the 'emmeans' v.1.7.5 package (Lenth 2022) and compared using the 'glht' function of the 'multcomp' v.1.4-19 package (Hothorn et al. 2008). To verify that any differences in results were not due to differences between software packages in the implementation of statistical routines, we also analyzed the data from *D. excentricus* Expt 1 using SPSS v.24 (IBM 2016). Similar values were produced by both packages.

To ensure our statistical conclusions were not particular to the approach that both we and Kacenas & Podolsky (2018) used, we conducted several additional analyses, including similar linear mixed-effects models that included covariate interaction terms as well as a simpler approach using ANOVA to compare PORL and SL adjusted for larval size by dividing each by BL. These analyses, which are detailed in the Supplement, all produced results similar to those of our primary analysis (Text S1, Tables S1–S5, Fig. S1 in the Supplement at www.int-res.com/articles/suppl/m694p001_supp.pdf).

3. RESULTS

3.1. Accuracy of larval density manipulations

In *Dendraster excentricus* Expt 1 as well as *Lytechinus pictus* Expts 1 and 2, count-control beakers for both the 0.05 and 0.25 larvae ml^{-1} densities contained an average of 80–100% of the intended number of larvae, suggesting that our manipulations of larval density were reasonably accurate. However, for *D. excentricus* Expt 2, the count-control beakers for both the 0.05 and 0.25 larvae ml^{-1} densities contained, on average, many fewer (46–48%) larvae than expected, suggesting that densities in these treatments were substantially lower than intended. We view this as a conservative error, providing an even stronger test of the ability of larvae to exhibit plasticity at low culture densities. Because embryos were hand-counted into the 0.015 larvae ml^{-1} treatments, we knew that at least initial numbers of larvae in those treatments were as intended.

3.2. Larval size and developmental stage

In both *D. excentricus* experiments, larvae developed longer BL and longer SL at the higher of the 2 food rations within each culture density (Fig. 2). The absolute length of the right postoral rod differed by food ration only in the second experiment at the highest culture density (0.25 larvae ml^{-1}), where larvae fed the lower food ration developed longer postoral rods. At 5 dpf, all photographed larvae of *D. excentricus* fed the high food ration (n = 50 in *D. excentricus* Expt 1; n = 75 in *D. excentricus* Expt 2) had developed the third (posterodorsal) pair of arms, whereas only 78% (Expt 1, n = 50) and 70.7% (Expt 2, n = 75) of larvae fed the low food ration had developed the third pair of arms (the remainder had developed only the first 2 pairs of arms).

The mean BL of larvae of *L. pictus* did not differ by food ration in *L. pictus* Expt 1 (5 dpf) and only dif-

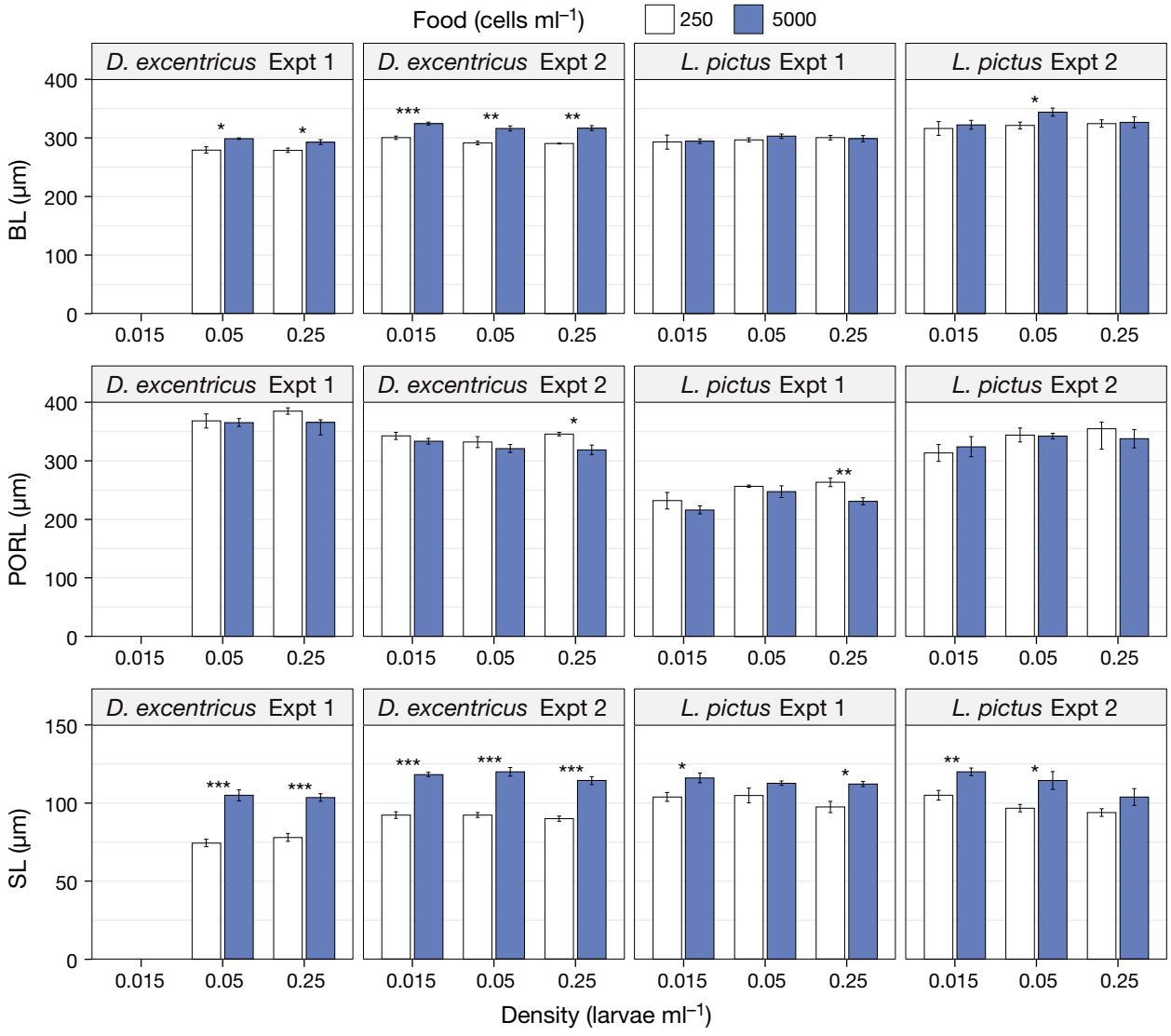

Fig. 2. Absolute body dimensions (mean ± SE) in all 4 experiments. *Dendraster excentricus* Expt 1 and *D. excentricus* Expt 2 are the experiments on *D. excentricus* with 2 and 3 larval density treatments, respectively; *Lytechinus pictus* Expt 1 and *L. pictus* Expt 2 are the experiments on *L. pictus* at 5 and 7 days post-fertilization, respectively. BL: body length; PORL: postoral rod length; SL: stomach length, all as defined in Fig. 1. Asterisks above bars: significant p-values for heteroscedastic *t*-tests comparing morphometric values between food rations: *p < 0.05; **p < 0.01; ***p < 0.001. No bars are shown for the culture density of 0.015 larvae ml^{-1} in *D. excentricus* Expt 1 because this treatment did not exist in that experiment

fered in *L. pictus* Expt 2 (7 dpf) in the 0.05 larvae ml^{-1} treatment, where larvae fed the higher food ration were significantly longer than those fed the lower food ration (Fig. 2). PORL was shorter for larvae fed the higher food ration only in *L. pictus* Expt 1 in the 0.25 larvae ml^{-1} treatment. SL was greater in larvae fed the higher food ration in the 0.015 and 0.25 larvae ml^{-1} treatments in *L. pictus* Expt 1 and in the 0.015 and 0.05 larvae ml^{-1} treatments in *L. pictus* Expt 2. In both *L. pictus* experiments, all photographed larvae had 2 pairs of arms (n = 141 in *L. pictus* Expt 1, n = 129 in *L. pictus* Expt 2).

3.3. Plasticity of PORL

Low-fed larvae had greater estimated marginal means of PORL than high-fed larvae in both *D. excentricus* experiments (Table 1). These differences were statistically significant (p < 0.05) in 4 out of 5 pairwise comparisons, the exception being the 0.05 larvae ml^{-1} treatment of *D. excentricus* Expt 1 (p = 0.071; Fig. 3). There was no effect of larval density on 1 (adjusted for BL) in *D. excentricus*, nor was there an interaction between larval density and food ration (Table 1).

Table 1. Type III ANOVAs for linear mixed-effects models of right postoral rod length (PORL) and stomach length (SL) of *Dendraster excentricus* and *Lytechinus pictus* using Satterthwaite's method. BL: body length; SS: sum of squares; MS: mean of squares; df_N: numerator degrees of freedom; df_D: denominator degrees of freedom. Significant results are in **bold:** $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$

Experiment	Source	SS	MS	df_N	df_D	*F*	p	Sig.
PORL								
D. excentricus Expt 1	**BL**	**36106.519**	**36106.519**	**1**	**91.592**	**36.973**	**<0.001**	***
	Density	2663.067	2663.067	1	16.156	2.727	0.118	
	Food	**15740.632**	**15740.632**	**1**	**22.628**	**16.118**	**<0.001**	***
	Density × food	221.368	221.367	1	16.105	0.227	0.640	
D. excentricus Expt 2	**BL**	**52797.295**	**52797.295**	**1**	**143.000**	**45.582**	**<0.001**	***
	Density	1068.517	534.259	2	143.000	0.461	0.631	
	Food	**48988.864**	**48988.864**	**1**	**143.000**	**42.294**	**<0.001**	***
	Density × food	3051.352	1525.676	2	143.000	1.317	0.271	
L. pictus Expt 1	**BL**	**48914.528**	**48914.528**	**1**	**128.743**	**41.651**	**<0.001**	***
	Density	**9592.387**	**4796.194**	**2**	**23.122**	**4.0839**	**0.030**	*
	Food	**15211.042**	**15211.042**	**1**	**23.097**	**12.952**	**0.002**	**
	Density × food	1626.930	813.465	2	22.614	0.693	0.511	
L. pictus Expt 2	**BL**	**53724.808**	**53724.808**	**1**	**110.882**	**32.555**	**<0.001**	***
	Density	6558.055	3279.027	2	22.269	1.987	0.161	
	Food	3628.311	3628.311	1	23.900	2.199	0.151	
	Density × food	1285.258	642.629	2	22.310	0.389	0.682	
SL								
D. excentricus Expt 1	**BL**	**262.357**	**262.357**	**1**	**94.649**	**4.916**	**0.029**	*
	Density	18.280	18.280	1	15.895	0.343	0.567	
	Food	**4482.653**	**4482.653**	**1**	**20.370**	**84.002**	**<0.001**	***
	Density × food	33.850	33.850	1	15.860	0.634	0.438	
D. excentricus Expt 2	**BL**	**1691.461**	**1691.461**	**1**	**142.953**	**23.338**	**<0.001**	***
	Density	392.222	196.111	2	24.470	2.706	0.087	
	Food	**8882.680**	**8882.680**	**1**	**50.201**	**122.559**	**<0.001**	***
	Density × food	72.065	36.032	2	23.109	0.497	0.615	
L. pictus Expt 1	**BL**	**5647.098**	**5647.098**	**1**	**134.000**	**25.919**	**<0.001**	***
	Density	1108.262	554.131	2	134.000	2.543	0.082	
	Food	**4052.755**	**4052.755**	**1**	**134.000**	**18.601**	**<0.001**	***
	Density × food	521.385	260.693	2	134.000	1.197	0.305	
L. pictus Expt 2	**BL**	**4074.020**	**4074.019**	**1**	**95.047**	**20.870**	**<0.001**	***
	Density	**3159.927**	**1579.964**	**2**	**22.990**	**8.094**	**0.002**	**
	Food	**3167.290**	**3167.290**	**1**	**24.980**	**16.225**	**<0.001**	***
	Density × food	61.177	30.588	2	23.078	0.157	0.856	

In *L. pictus* Expt 1, but not *L. pictus* Expt 2, food ration affected estimated marginal means of PORL (Table 1). However, the difference in PORL (adjusted for BL) only approached significance (p = 0.053) in the 0.25 larvae ml^{-1} treatment of this experiment, where low-fed larvae had longer postoral rods than did high-fed larvae (Fig. 3). Larval density also affected estimated marginal means of PORL in *L. pictus* Expt 1, but not *L. pictus* Expt 2 (Table 1), though there were no pairwise differences between densities (Table 2). Adjusted PORL showed no evidence of a statistical interaction between larval density and food ration in either *L. pictus* experiment (Table 1).

3.4. Plasticity of SL

Food ration had a significant effect on estimated marginal means of SL in both *D. excentricus* experiments (Table 1). SL was greater in the higher food ration at every density level in both experiments when adjusted for BL (Fig. 4). Larval density had no effect on the estimated marginal means of SL in *D. excentricus* nor was there an interaction between larval density and food ration (Table 1).

While food ration had a significant effect on estimated marginal means of SL in both *L. pictus* experiments, the only significant pairwise differences between food rations (p = 0.017) occurred in the

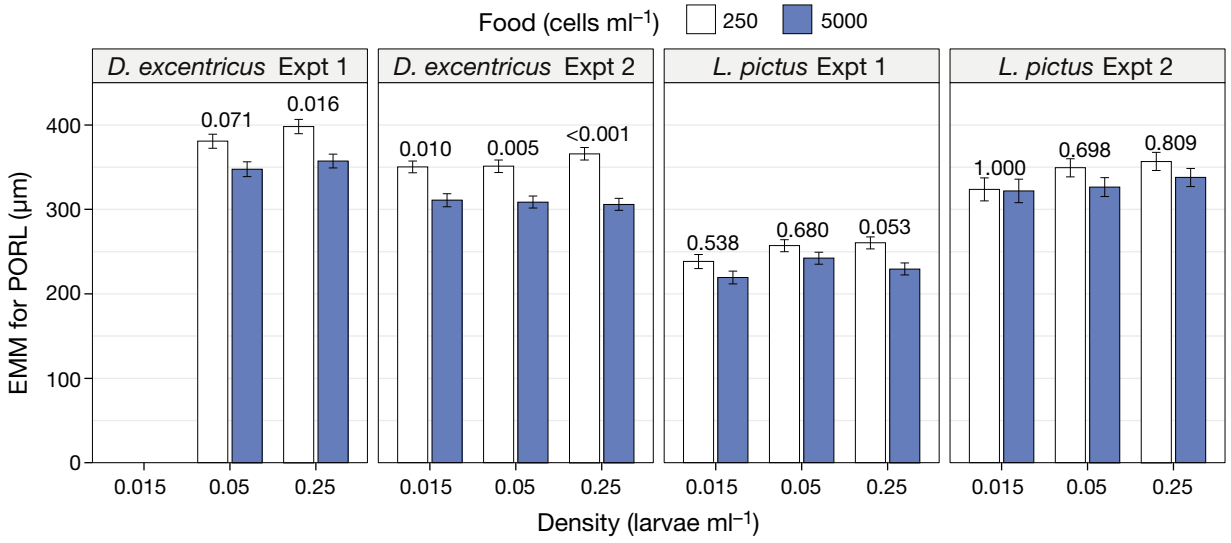

Fig. 3. Estimated marginal means (EMM) for postoral rod length (PORL). Error bars: ±SE. Numbers above a pair of bars: p-values from Kenward-Roger's *F*-test with Tukey's adjustment for 4 estimates (*Dendraster excentricus* Expt 1) or 6 estimates (*D. excentricus* Expt 2, *Lytechinus pictus* Expt 1, *L. pictus* Expt 2). No bars are shown for the culture density of 0.015 larvae ml^{-1} in *D. excentricus* Expt 1 because this treatment did not exist in that experiment

Table 2. Tukey contrasts between density levels in the linear mixed-effects models which showed a significant effect of *Lytechinus pictus* larval density. Significant results are in **bold:** *p < 0.05. PORL: postoral rod length; SL: stomach length

Experiment	Contrast	Estimate	SE	*z*	p	Sig.
PORL						
L. pictus Expt 1	0.05 vs. 0.015 ml^{-1}	18.827	10.797	1.744	0.189	
	0.25 vs. 0.015 ml^{-1}	22.202	10.864	2.044	0.102	
	0.25 vs. 0.05 ml^{-1}	3.375	10.023	0.337	0.939	
SL						
L. pictus Expt 2	0.05 vs. 0.015 ml^{-1}	−9.051	4.912	−1.843	0.155	
	0.25 vs. 0.015 ml^{-1}	**−12.679**	**4.928**	**−2.573**	**0.027**	*
	0.25 vs. 0.05 ml^{-1}	−3.628	4.346	−0.835	0.681	

dense cultures (0.25 larvae ml^{-1}) in *L. pictus* Expt 1. Estimated marginal means of SL differed among density treatments in *L. pictus* Expt 2 ($p = 0.002$) but not in *L. pictus* Expt 1 ($p = 0.082$; Table 1). For *L. pictus* Expt 2, larvae in the 0.015 larvae ml^{-1} cultures had longer stomachs than those in the 0.25 larvae ml^{-1} cultures ($p = 0.027$; Table 2). Adjusted SL showed no evidence of a statistical interaction between larval density and food ration in either *L. pictus* experiment (Table 1).

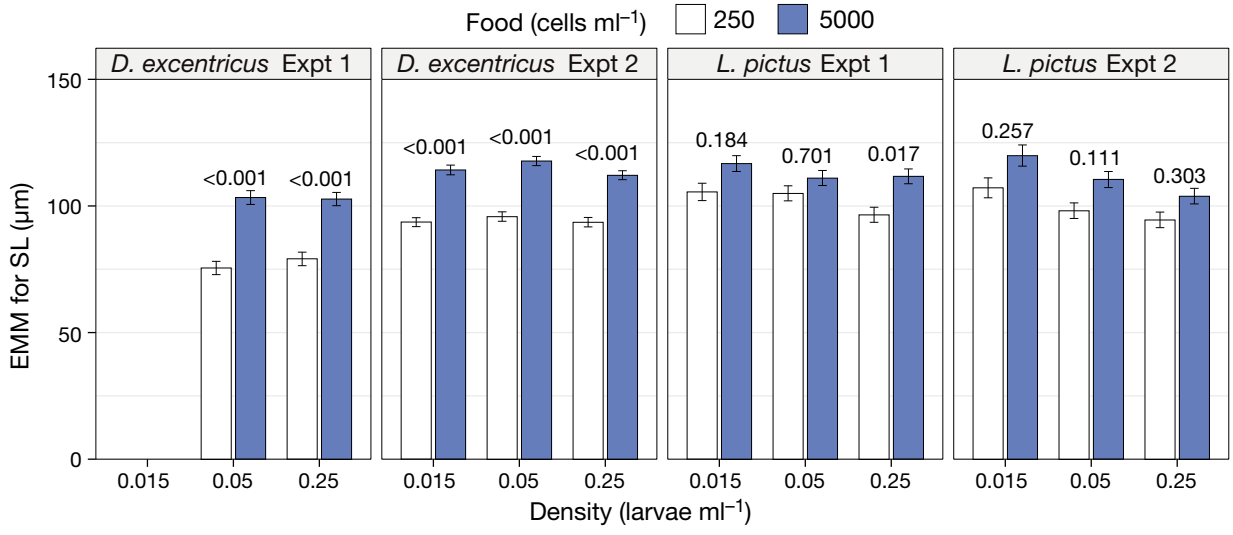

Fig. 4. Estimated marginal means (EMM) for stomach length (SL). Error bars and p-values as in Fig. 3

3.5. Tradeoff between PORL and SL

Estimated marginal means of PORL and SL generally displayed opposite responses to food level. For both species and at almost all culture densities, larvae fed the high food ration had short PORL and long SL, whereas larvae fed the low food ration had long PORL and short SL (Fig. 5). The only exception was the lowest density treatment in *L. pictus* Expt 2, where food ration affected PORL (with high-fed larvae having short PORL) but not SL.

4. DISCUSSION

Kacenas & Podolsky (2018) found that larvae of the sand dollar *Dendraster excentricus* expressed food-conditioned plasticity of feeding structure size only when reared at a culture density that was much higher than likely larval densities in the plankton. This interaction between food ration and larval density did not appear to be a consequence of food limitation imposed by increased competition for food, as plasticity occurred in larvae reared at high densities even when most larvae in the culture were non-feeding heterospecifics. This finding suggests that the many laboratory studies identifying feeding structure plasticity in echinoid larvae (McAlister & Miner 2018) may not be particularly relevant to the conditions in which larvae are actually found in nature, as in almost all such studies larvae were cultured at relatively high densities. However, our experiments showed that larvae can exhibit phenotypic plasticity at low culture densities, suggesting that laboratory experiments may indeed be useful models of plasticity that may occur in field conditions.

4.1. *D. excentricus*

Our results showed both similarities to and differences from those of Kacenas & Podolsky (2018). Like Kacenas & Podolsky (2018), in one of our experiments (*D. excentricus* Expt 1) adjusted larval PORL clearly showed a plastic response to food ration in cultures of 0.25 larvae ml^{-1} ($p = 0.016$), but not in cultures of 0.05 larvae ml^{-1} ($p = 0.071$; Fig. 3). However, in *D. excentricus* Expt 2, larvae developed longer postoral rods (adjusted for BL) in both the culture densities used by Kacenas & Podolsky (2018) as well as in a third, even lower culture density (0.015 larvae ml^{-1}) closer to larval densities likely to occur in nature (Fig. 3). Pairwise comparisons between high- and low-fed larvae produced lower p-values in higher culture densities. While this gives the impression that larvae showed a stronger response to food ration at higher culture densities, this interpretation is not borne out in statistical tests, as the ANOVAs for adjusted PORL revealed no interaction between food ration and density in either *D. excentricus* experiment ($p = 0.640$, $p = 0.271$; Table 1), indicating that the ability of larvae to respond plastically to food availability did not depend on their culture density.

SL data reinforces the evidence that food-conditioned phenotypic plasticity can occur at low culture densities in *D. excentricus*: when adjusted for BL, SL

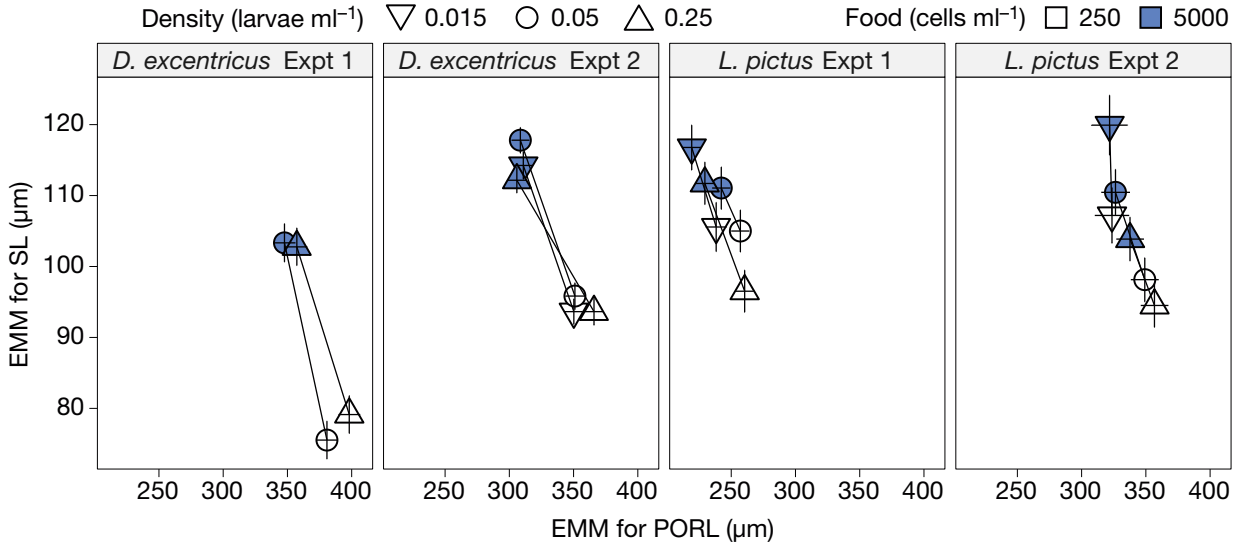

Fig. 5. Relationship between the estimated marginal means (EMM) of postoral rod length (PORL) and stomach length (SL) in all 4 experiments. Lines connect treatments that differ only in food ration. Error bars: ±SE

was greater in larvae fed the higher of 2 food rations at all culture densities in both experiments (Fig. 4). Further, at all culture densities in both experiments, SL appeared to trade off against PORL (Fig. 5). This pattern is consistent with prior work suggesting that echinoid larvae are subject to a tradeoff between investment in the length of postoral arms (ephemeral larval structures) and the size of the stomach (which carries over to the juvenile stage) when showing a plastic response to food levels (e.g. Strathmann et al. 1992, Miner 2005, Kacenas & Podolsky 2018).

Our experiments thus showed substantial evidence of food-conditioned phenotypic plasticity in PORL in *D. excentricus* even at the lowest culture densities tested. Our results may differ from those of Kacenas & Podolsky (2018) for a variety of reasons. One potential reason is that we reared larvae of *D. excentricus* at a higher temperature than they did (16°C for all stages rather than 10–14°C from fertilization to 3 dpf and 12–14°C from 3–8 dpf). We expected that this would lead to more rapid development in our cultures, and to account for this difference, we photographed larvae earlier than did Kacenas & Podolsky (2018) (5 dpf rather than 8 dpf). Therefore, we may have examined larvae at a slightly different stage of development than Kacenas & Podolsky (2018). The warmer temperature we used also has physiological consequences for larvae, leading to a higher metabolic rate and thus higher demand for food. We fed larvae the same alga at the same 2 rations as Kacenas & Podolsky (2018) rather than increasing food rations to compensate for the higher metabolic demands of our larvae, which may have resulted in more severe food limitation, potentially eliciting a stronger plastic response. The viscosity of seawater varies with temperature, which may impact swimming and feeding performance (Podolsky & Emlet 1993, Podolsky 1994). Given the broad range of variables influenced by temperature, it is unsurprising that echinoid larvae can respond to the same food rations in different ways depending on temperature (García et al. 2015). While the effects of temperature on food-conditioned plasticity in echinoid larvae have rarely been studied, one study found greater morphological responses to food ration in larvae of *Strongylocentrotus droebachiensis* raised at 9°C than those raised at 3 or 6°C (Hart & Scheibling 1988).

It is also possible that the genetic diversity of larvae influences whether and to what degree plasticity occurs. McAlister & Miner (2018) observed that many experiments on feeding structure plasticity use a single full-sibling family of larvae, making generalization of results to the population or species level difficult. In each of our experiments, we used a population of larvae derived in equal proportions from 3 unique families. Kacenas & Podolsky (2018) did not describe the parentage of their larvae; if they studied a less diverse larval population (e.g. a single full-sibling family) than we did and if the degree of plasticity varies among families, then the population of larvae they created may have had a lesser capacity for plasticity. Increasing the genetic diversity of larvae within an experiment (as we have done) should reduce the risk of obtaining larvae which all have low capacity for plasticity. On the other hand, our approach has some disadvantages. If there are non-plastic genetic effects on larval proportions, diversity might weaken the plasticity signal. Mixing families within treatments may also complicate interpretation of larval morphology: if families differ both in form and in mortality rate, then the population of larvae will, with time, be biased towards forms typical of low-mortality families. Finally, aside from potential differences in the parentage of larvae studied in these experiments, the source populations of *D. excentricus* that we and Kacenas & Podolsky (2018) studied were separated by ~2000 km, and thus may have had genetic differences causing them to differ in their expression of phenotypic plasticity.

One limitation with our data is that larvae in the different food rations reached slightly different developmental stages during experiments. Larvae of *D. excentricus* fed the higher food ration all developed 3 pairs of arms by the time of measurement, whereas only 70.7–78% of larvae fed the lower food ration in both experiments reached that developmental stage. It is unclear if Kacenas & Podolsky (2018) also faced this problem, as they did not report what proportion of their larvae were at which stage. Comparisons of larvae of different developmental stages is not ideal, as larvae may vary in their investment in growth of particular body parts as a function of developmental stage. This is a widespread issue in studies of phenotypic plasticity in echinoid larvae. While it may not be possible to ensure that larvae reared on different rations are measured at identical stages of development, Sewell et al. (2004) suggested that other statistical approaches, such as principal component analysis, may be helpful in disentangling stage from plasticity.

4.2. *Lytechinus pictus*

We expected larvae of *Lytechinus pictus* to show food-conditioned plasticity of feeding structures be-

cause its congener, *L. variegatus*, displayed such plasticity in a previous study (Boidron-Metairon 1988). Contrary to our expectations, the evidence for plasticity in *L. pictus* was equivocal. Experiment-wide, food ration affected adjusted PORL in *L. pictus* Expt 1 ($p = 0.002$) but not in Expt 2 ($p = 0.151$; Table 1). In these experiments, 5 out of 6 pairwise comparisons between high- and low-fed larvae followed the pattern we would expect for a plastic response, with higher estimated marginal means of PORL in low-fed larvae (Fig. 3). However, in only one contrast (the 0.25 larvae ml^{-1} density in *L. pictus* Expt 1, $p = 0.053$; Fig. 3) was this difference in length even marginally significant.

The SL data present a similar picture. In both experiments, food ration affected adjusted SL ($p < 0.001$; Table 1), and estimated marginal means followed the pattern we would expect for plasticity, being greater for high-fed larvae than low-fed larvae at each density (Fig. 4). However, these differences were again not significant, with one exception: the 0.25 ml^{-1} treatment in *L. pictus* Expt 1 ($p = 0.017$; Fig. 4). As with *D. excentricus*, PORL and SL generally showed opposite responses to food availability, suggesting a trade-off consistent with prior work on plasticity (Fig. 5).

We believe these results suggest that larvae of *L. pictus* might exhibit the same phenotypic plasticity of feeding structure size that occurred in *D. excentricus*, but our post hoc tests lacked the statistical power to detect the relatively minor phenotypic differences between high- and low-fed larvae that were detected by experiment-wide ANOVAs. Low statistical power is a pervasive challenge for experiments on larval feeding plasticity (McAlister & Miner 2018). In both of our *L. pictus* experiments, our qualitative observations suggested that larvae varied more in form than did those of *D. excentricus*, despite all being at the 4-arm pluteus stage. High morphological variability was also true in other *L. pictus* cultures in our lab, even where full siblings shared the same container (B. Steiner pers. comm). This variability in larval form may weaken the plasticity signal, if one exists.

One prior study has suggested that embryos of *L. pictus* might exhibit plasticity in PORL in response to nutritional conditions (Shilling 1995). However, that study is not directly comparable to our study or others discussed here. In Shilling's (1995) experiments, embryos were reared from fertilization to 2 dpf in FSW alone or FSW supplemented with additional dissolved organic matter in the form of algal exudate, amino acids, sugar, or palmitic acid; no particulate food was available. At the end of this period, embryos had reached the late gastrula or early prism stage, judging from images in Fig. 2 of Shilling (1995). Embryos reared in the presence of supplemental amino acids and palmitic acid developed absolutely shorter postoral skeletal rods than those reared in FSW alone. Though it is unclear exactly how this finding relates to that of more typical studies of food-conditioned plasticity, where study subjects are fed particulate food and examined at post-embryonic stages, it does suggest that larvae of *L. pictus* have the capacity to modulate the length of their postoral skeletal rods in response to environmental conditions.

We note that even for *L. variegatus*, one of the species in which phenotypic plasticity in postoral arm length was first reported (Boidron-Metairon 1988), not all studies have found food-conditioned plasticity. McAlister & Miner (2018) reported that in studies of 2 North Carolina populations of *L. variegatus carolinus*, food-conditioned plasticity of larval arm length was apparent in one but seemingly absent in the other. Buitrago et al. (2005) also did not find plasticity of larval length in *L. variegatus*, though we note their measurement of length (as the sum of the lengths of the body and longest arm) is poorly suited to detecting plasticity. In the context of these data on *L. variegatus*, it is perhaps unsurprising that our experiments on *L. pictus* produced equivocal results. It is possible that larvae of *L. pictus* exhibit more dramatic food-conditioned phenotypic plasticity at a different point in development than our experiments sampled. This may also be the case for *L. variegatus*, which displayed plasticity at 4 dpf but not later in one study (Boidron-Metairon 1988), as well as for at least one other urchin, *Evechinus chloroticus* (Sewell et al. 2004).

As our *L. pictus* results are only suggestive of plasticity, we can only make tentative conclusions about the relationship between culture density and expression of phenotypic plasticity in this species. Paralleling the results of Kacenas & Podolsky (2018), we found that pairwise comparisons of adjusted PORL produced the lowest p-values at the highest culture density (Fig. 3), and for *L. pictus* Expt 1, the ANOVA revealed an effect of culture density on adjusted PORL ($p = 0.030$; Table 1). However, as was the case for our *D. excentricus* data, the adjusted PORL ANOVAs revealed no interaction between food ration and density in either *L. pictus* experiment ($p = 0.511$, $p = 0.682$; Table 1), indicating that the ability of larvae to respond plastically to food availability did not depend on their culture density.

Similarly, for adjusted SL, the pairwise difference between high- and low-fed larvae yielded the lowest

p-value (0.017) in the highest culture density treatment (0.25 larvae ml^{-1}) in *L. pictus* Expt 1 (Fig. 4), consistent with Kacenas & Podolsky's (2018) finding of greater plastic response at higher culture densities. We also note there was an experiment-wide effect of density on adjusted SL in *L. pictus* Expt 2 ($p = 0.002$; Table 1). However, as with the PORL data, the ANOVAs revealed no interaction between ration and density for adjusted SL in either experiment ($p = 0.305$, $p = 0.856$; Table 1), indicating that the low p-value of the high-density treatment in one experiment should not be interpreted as evidence of a stronger plastic response. Additionally, we found evidence of a tradeoff between PORL and SL at all culture densities in *L. pictus* Expt 1, and at both the 0.05 and 0.25 larvae ml^{-1} densities in *L. pictus* Expt 2 (Fig. 5). This contrasts with Kacenas & Podolsky's (2018) data, which showed this pattern only at the highest culture density (0.25 larvae ml^{-1}).

Thus, while the lack of clear evidence for plasticity in *L. pictus* at any density precludes direct comparison with Kacenas & Podolsky's (2018) results, our data on this species do not suggest that high culture densities lead to spurious findings of plasticity.

4.3. Conclusions

In a key study, Kacenas & Podolsky (2018) tested the assumption that larvae reared in the laboratory in relatively dense cultures respond to food limitation in the same way that they would in the plankton, where larvae generally occur at very low densities. They found that culture density affected the expression of phenotypic plasticity in larvae of *D. excentricus*, with larvae reared at high density showing a plastic response to food limitation but larvae reared at low density not showing such a response. This suggests that the results of prior studies on food-conditioned phenotypic plasticity in echinoid larvae, almost all of which were carried out at high culture densities, may not be particularly relevant to understanding how larvae develop in nature.

Our data, however, show that larvae of *D. excentricus* can express food-conditioned phenotypic plasticity when reared in the laboratory at low densities approaching those they presumably experience in the plankton. It remains possible that culture density affects the magnitude of plastic responses, however, although this requires additional study. While we did not find strong evidence of plastic responses at any culture density in larvae of a second echinoid, *L. pictus*, our results suggest that rearing them at the relatively high densities typical of laboratory culture does not exaggerate their expression of plasticity. Together, our results suggest that existing knowledge on the distribution, mechanisms, and functional consequences of feeding structure plasticity in echinoid larvae remains ecologically relevant, even though almost all studies of plasticity have been carried out in high-density cultures unrepresentative of natural conditions. This conclusion is strengthened by the one study we are aware of that sought evidence of food-conditioned phenotypic plasticity in larvae captured from the field. Fenaux et al. (1994) found that field-collected larvae of the echinoid *Paracentrotus lividus* had longer arms relative to body size in the autumn, when less phytoplankton was present, than in the spring, when more phytoplankton was present. Additional field studies of larvae are clearly needed in order to better understand the causes, frequency, and significance of food-conditioned phenotypic plasticity in nature.

The results of prior laboratory studies of phenotypic plasticity carried out at unnaturally high culture densities may thus still be useful in understanding how echinoid larvae develop in nature. However, it is clear that culture density affects many aspects of larval physiology, growth, and development (Kacenas & Podolsky 2018, Hodin et al. 2019). As noted by Kacenas & Podolsky (2018), culture density must be carefully considered in the design of any laboratory experiments aimed at elucidating ecologically relevant aspects of larval biology.

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