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Insulin/IGF Signaling and Life History Traits in Response to Food Availability and Perceived Density in the Cnidarian *Hydra vulgaris*

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Insulin/insulin-like growth factor signaling (IIS) is thought to be a central mediator of life history traits, but the generality of its role is not clear. Here, we investigated mRNA expression levels of three insulin-like peptide genes, the insulin-like receptor *htk7*, as well as several antioxidant genes, and the heat-shock protein *hsp70* in the freshwater cnidarian *Hydra vulgaris*. Hydra polyps were exposed to a combination of different levels of food and perceived population density to manipulate life history traits (asexual reproduction and oxidative stress tolerance). We found that stress tolerance and the rate of asexual reproduction increased with food, and that these two effects were in significant interaction. Exposing animals to high perceived density resulted in increased stress tolerance or reduced reproduction only on lower food levels, but not on high food. The insulin-like receptor *htk7* and the antioxidant gene catalase were significantly upregulated in the high density treatments. However, the expression level of insulin-like peptide genes, most antioxidant genes, and *hsp70* were not affected by the experimental treatments. The higher expression level of *htk7* may suggest that animals maintain a higher level of preparedness for insulin-like ligands at high population densities. However, the lack of difference between food levels suggests that IIS is not involved in regulating asexual reproduction and stress tolerance in hydra, or that its role is more subtle than a simple model of life history regulation would suggest.

Key words: antioxidant genes, asexual reproduction, Cnidaria, oxidative stress, resource allocation trade-offs

INTRODUCTION

Trade-offs between life-history traits that contribute to survival and reproduction are key assumptions of models of life history evolution (Stearns, 1992; Roff, 1993). As all such life history components have energetic costs, maximizing them simultaneously under limited resources is not possible, and trade-offs become inevitable (Partridge et al., 2005). Understanding the mechanisms mediating resource allocation between self-maintenance and reproduction is a central challenge in evolutionary biology.

Despite the established diversity of life history trade-offs, little is known about their underlying physiological and molecular machineries (Flatt and Heyland, 2011). The consistent correlation among a set of life-history traits and the lack of some combinations in nature (Promislow and Harvey, 1990; Charnov, 1993; Ricklefs and Wikelski, 2002) suggests a shared pleiotropic control mechanism behind the variation of life history strategies (Reding et al., 2016). Insulin/insulin-like growth factor signaling (IIS) pathway is an evolutionarily conserved regulatory system for such a control mechanism, since it plays important roles in shaping life histories both in vertebrates and invertebrates (Tatar et al., 2003). Components of IIS regulate development, longevity, metabolism and reproduction in diverse animals (e.g. *Caenorhabditis elegans* (Ogg and Ruvkun, 1998), *Drosophila melanogaster* (Garofalo, 2002) and *Mus musculus* (Fantin et al., 2000)). Single mutations in members of the IIS pathway have a great influence on animal life histories; they result in extended lifespan (Kenyon et al., 1993; Bartke, 2011), growth deficiency or dwarfism (Eigenmann et al., 1984; Garofalo, 2002) or reduced fertility and viability (Tissenbaum and Ruvkun, 1998).

Insulin is a potent metabolic and growth-promoting hormone with multiple effects on cells: it stimulates glucose, protein and lipid metabolism as well as RNA and DNA synthesis by modifying the activity of several enzymes and transport processes (Kahn et al., 1993). Furthermore, IIS positively regulates reproduction by promoting vitellogenesis and oocyte growth in insects (Badisco et al., 2013) and follicle development in mammals (Burks et al., 2000). IIS

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also influences cellular maintenance processes; most importantly, it regulates the expression of reactive oxygen species scavengers and other components of the cellular defense system (e.g. the antioxidant enzymes superoxide dismutase and catalase (Vanfleteren, 1993)) which function to inhibit or delay the oxidation of substrates (Finkel, 2003; Matkowski, 2008). Reduced activity of the insulin-signaling pathway is therefore associated with higher tolerance of oxidative stress and extended longevity (Hsin and Kenyon, 1999; Holzenberger et al., 2003; Broughton et al., 2005; Flatt et al., 2011).

These pleiotropic effects of the insulin IIS pathway are thought to have appeared early during the evolution of the first metazoans (Pertseva and Shpakov, 2002). Components of the IIS pathway have been documented in basal animals, such as sponges (Skorokhod et al., 1999) and cnidarians (Steele et al., 1996; Bridge et al., 2010). However, our current knowledge about how these mechanisms may govern life histories comes from only a handful of model organisms. The investigation of these mechanisms in basal metazoans may therefore provide further insights to the evolution of life histories.

Freshwater hydras are widespread cnidarians that have been used as model organisms in aging and life-history studies for decades (Lenhoff, 1983; Martínez and Bridge, 2012; Tomczyk et al., 2015). The genus *Hydra* is especially interesting because they show constant fertility and low mortality under stable conditions in the laboratory (Martínez, 1998; Schaible et al., 2015). Under natural conditions, their life histories are highly diverse in many aspects like reproductive modes (Lenhoff, 1983; Schuchert, 2010), life cycles (Tomczyk et al., 2015) or responses to environmental changes (Schaible et al., 2011; Kaliszewicz and Lipińska, 2013). Members of *Hydra* are capable of both asexual and sexual reproduction, and at least in one species (*Hydra oligactis*) sexual reproduction is sometimes followed by a senescence-like degeneration characterized by a progressive decline in food capture rate, reproduction and an increase in mortality (Yoshida et al., 2006; Tökölyi et al., *in press*). Asexual reproduction in hydra occurs by budding, in which a region of the parent body becomes remodeled into a small but complete animal (Otto and Campbell, 1977). Bud tissue arises primarily by cell proliferation of adjacent parent cells which depends on the resources available to the parent animal. Food availability has been shown to have a significant effect on life history strategies of *Hydra* species; at different food levels, low rates of reproduction and high stress tolerance or, conversely, nearly the complete opposite can occur in different species (Tökölyi et al., 2016). Life history traits are also adjusted to a number of environmental conditions, such as the predictability of food (Schaible et al., 2011), or the local population density (Thorp and Barthalmus, 1975; Tökölyi et al., 2014). If hydra are kept in a medium which previously contained a high number of individuals, the rate of asexual reproduction declines proportionally with increasing population density (Thorp and Barthalmus, 1975).

In the present study, we combined two of these effects to alter life history traits (asexual reproduction and oxidative stress tolerance) in *Hydra vulgaris* polyps. We manipulated food availability and perceived density in a factorial design to test how resource availability influences the documented effects of population density on life-history decisions (reproduction vs. self-maintenance) (van Noordwijk and de Jong, 1986). By using genetically identical animals, we minimized individual variation in resource allocation strategies, while keeping resource availability and population density under experimental control. Within this experimental setup we examined whether differences in life history traits are associated with altered expression of genes encoding hydra insulin-like peptides (*ilp1*, *ilp2*, *ilp3*; Fujisawa, 2008), the Hydra insulin-like receptor (*htk7*; Steele et al., 1996), and several genes involved in cellular maintenance processes: the heat-shock protein (*hsp70*) and four antioxidant enzymes: superoxide-dismutase (*sod*), catalase (*cat*), glucose-6-phosphate dehydrogenase (*g6pd*) and glutathione peroxidase (*gpx*), which were previously shown to be upregulated in *H. vulgaris* exposed to heat stress (Gellner et al., 1992) or oxidative stress by pesticide toxaphane (Woo et al., 2012). The five examined enzymes related to cellular maintenance are likely regulated by IIS, according to previous studies in diverse organisms e.g. HSP70, SOD and CAT in *Caenorhabditis elegans* (Dalley and Golomb, 1992; Vanfleteren, 1993), GPX in mice (Marinkovic et al., 2007), and G6PD in humans (Zhang et al., 2010). We predicted that *ilp* and *htk7* expression levels would increase with food availability, as *ilp*s are part of a nutrient-sensing pathway (Tatar et al., 2003). We also predicted that the expression level of *ilp*s and *htk7* would be lower, while that of cellular maintenance genes would be higher at high perceived population density because under these conditions reproduction is reduced while stress tolerance is enhanced in *Hydra* (Tökölyi et al., 2014). Regarding the relationship between food availability and cellular maintenance genes, we had two hypotheses. (1) These genes are negatively regulated by the IIS pathway in other animals; therefore, if IIS is activated at higher food levels in *Hydra*, a negative relationship between food amount and expression of cellular maintenance genes would be expected. (2) Stress tolerance in *Hydra vulgaris* is positively related to food (Tökölyi et al., 2016), hence a positive relationship between food level and the activation of cellular maintenance genes might also be predicted.

MATERIALS AND METHODS

Hydra strain and culture conditions

The experimental animals were descendants of a single polyp collected from the river Hortobágy (Eastern Hungary, 48.35° N, 21.27° E) in June 2014. We established a strain from this polyp by asexual propagation. Hydras were kept in a Memmert ICP 700 climate chamber at constant temperature (20°C) and photoperiod (12 h dark:12 light cycle). For culturing, we used a standard hydra medium containing 1.0 mM $CaCl_2$, 0.1 mM $MgCl_2$, 0.03 mM KNO_3, 0.5 mM $NaHCO_3$ and 0.08 mM $MgSO_4$. The mass culture was kept in glass trays and fed with a moderate amount (2 ml) of freshly hatched *Artemia* nauplii suspension. We dosed nauplii from a dense layer at the bottom of a test tube with an automatic pipette as described previously (Tökölyi et al., 2016).

Experimental design

At the start of the experiment, we set up six factorially arranged experimental groups by changing the perceived density (low or high perceived density treatment) and the quantity of food (low, medium or high food level treatment). On day 1 of the experiment, we moved

animals to six-well microplates, each well containing three polyps in 5 ml of hydra medium. Polyps were randomly assigned to plates. Each plate contained units of a single experimental group. Single plates from each experimental group were stacked in a random order and stacks were distributed evenly in the climate chamber to equalize any differences due to temperature within the climate chamber. 144 animals in eight plates were used for each experimental group (altogether 864) but nine individuals died or were accidentally lost during the 16 days of the study. Animals were fed every other day as described above according to their food level treatment: 10 μl (low), 30 μl (medium) and 90 μl (high) of *Artemia* (1 μl of *Artemia* suspension contains approximately 7–8 nauplii (Tökölyi et al., 2016)). Approximately one hour after feeding, when animals ingested food, we changed the medium in each well. During the experiment, we fed the animals in the mass culture with 0.5 ml brine shrimp suspension and changed their culture medium every other day.

Manipulating perceived density

To simulate high population density, we kept experimental animals in medium derived from the mass culture (crowded culture medium), which was prepared by keeping the mass culture in the medium for two days prior to usage. The mass culture contained ~ 800 hydras in 450 ml medium, therefore perceived density was ~ 1.8 individuals/ml (high) in the manipulated group. Control groups were kept in fresh culture medium; since there were $n = 3$ hydras in 5 ml medium, density in this case was 0.6 individuals/ml (low) in the control group. This difference in perceived density has been shown to cause decreased budding in hydra (Thorp and Barthalmus, 1975).

Quantifying the rate of asexual reproduction

We quantified the rate of asexual reproduction by counting the number of buds detached from experimental individuals during two-days intervals. In the course of the experiment, we recorded the actual reproduction rate before feeding approximately at the same time of day on each occasion, and removed the detached buds after recording. Sexual reproduction did not occur in this strain under our culturing conditions.

Measuring stress tolerance

We quantified stress tolerance on day 16 (after counting buds and moving experimental animals to fresh medium), by exposing a subset of the animals ($n = 207$) to hydrogen-peroxide (4.38 μM, 25 μl 3% H_2O_2 in 5 ml of hydra medium). The measurement of stress tolerance was based on a morphological scale based on Quinn et al. (2012), which we modified by including an additional level in order to refine the scale (Tökölyi et al., 2016). According to this scheme, stress tolerance had nine levels (0–8), based on the contraction and disintegration of the trunk and the tentacles. A score of 0 means an entirely disintegrated polyp, and a score of 8 means a fully intact animal. Stress tolerance level was determined 24 h after the addition of H_2O_2 by the same person (FS). Recording measurements by the same person was necessary to avoid bias arising from different rating of morphological characteristics by different raters.

Real-time quantitative PCR analysis

12 animals (not included in the stress tolerance test above) were homogenized using a pellet pestle in 1 ml Trizol (Life Technologies) to obtain one biological sample, with three biological samples in each experimental group. Total RNA was isolated according to manufacturer's instructions on day 16 (i.e. after the experiment was finished). 1 μg of total RNA samples were transcribed to cDNA using High Capacity cDNA Reverse Transcription kit (Life Technologies) according to manufacturer's protocol, then cDNA samples were diluted by five times with nuclease free water and 5 μl of the diluted cDNA samples were used for RT-qPCR measurements. LightCycler 480 SYBR Green I Master mix (Roche) was used and the measurements were performed on LC480 QPCR instrument (Roche). Relative gene expression levels of genes (indicated in Table 1) were calculated by using the ΔC_T method; the housekeeping gene *tuba*1 was used as reference gene. We used published primers (*htk7* and *hsp70* form Steele et al., 1996; *tuba1*, *cat*, *sod*, *gpx*, *g6pd* from Woo et al., 2012) or designed primers ourselves, based on sequences deposited in GenBank (http://www.ncbi.nlm.nih.gov/genbank/). Primers were designed using the Primer3 tool (Untergasser et al., 2012), with default parameters. The GenBank accessions used for primer design were: GU219979 (for *ilp1*), GU219980 (for *ilp2*) and GU219981 (for *ilp3*). Since these are short mRNA sequences, we were not able to design intron-spanning primers (i.e. primers that only amplify mRNA but not genomic DNA). To test for potential genomic contamination, we used the *hsp70* primers, as described in Steele et al. (1996). These primers flank two introns and thus amplify products of different size from genomic DNA and mRNA (669 and 402 bp, respectively). Genomic contamination was not detectable in our samples.

Statistical analysis

To analyze asexual reproduction rate, sampling units were individual wells i.e. total number of buds produced by three animals during two days. Because this was a count variable, we used Generalized Linear Mixed Models (GLMMs) with Poisson error distribution. Oxidative stress tolerance scores were analyzed by Cumulative Link Mixed Models (CLMMs). This was needed because stress tolerance code is an ordinal variable, i.e. the differences between the levels of stress tolerance codes do not mean equal intervals. To test the effect of the two treatment types (food treatment and density treatment) and their interactions, we used Likelihood Ratio Tests (LRTs), by comparing models with the two treatments and their interaction to the model without the interaction, or models containing individual treatments to null models. We included plate ID as a random effect, to control for the possibility that animals within a plate might be more similar to each other than expected by chance (because of the shared position in the climate

Table 1. Primer sequences used for real-time quantitative PCR to quantify transcript levels of insulin-like peptide genes (*ilp1*, *ilp2*, *ilp3*), the Hydra insulin-like receptor gene *htk7*, four antioxidant genes (*sod*, *cat*, *g6pd* and *gpx*), the heat shock protein gene *hsp70* and the housekeeping gene *tuba1*.

Gene	Forward primer	Reverse primer	Source
htk7	AGTACTTAATTTTGTGCTCAGTAA	GTAACTTTCGCTTTTCATATAGAT	Steele et al. (1996)
ilp1	ACGCACAAGCATTATGTGGA	ATTCTTCATCGGCGTTGTCT	This study
ilp2	TCTGCGGATGATTACGACAA	AACTTTGCCACTGGATTTGG	This study
ilp3	TCGCACCGTTCGTTTCTATT	GCTTGTCGCTTGGTTACCTC	This study
hsp70	CACGGAAAAGTTGAAATAATTGCT	CTTGACGTTGAGAATCATTAAAGT	Steele et al. (1996)
cat	GCTCCAAACTACTTCCCTAACAG	GCTCATCTATCGCTTCATTT	Woo et al. (2012)
g6pd	GCATTGCCACCATCTGTATTCA	GCAAACCTTAGCACCATTAT	Woo et al. (2012)
gpx	TCGATATCTGGAACCAATGACAAA	CGAGGCGCCCACTATGACTT	Woo et al. (2012)
sod	TCAGTTTGGGGATTATTCAGGTG	TCCAGCATTTCCGGTAGTTTTG	Woo et al. (2012)
tuba1	TTGATGAAATACGCACAGGAACA	CCACCAAAGGAATGAAAAAT	Woo et al. (2012)

chamber). LRTs were followed by *post-hoc* tests with Bonferroni-Holm correction. All analyses were performed in the R Statistical Environment (R Core Team, 2016), employing the *ordinal* package (Christensen, 2015) to implement CLMMs, *lme4* (Bates et al., 2015) for GLMMs and the *lsmeans* (Lenth, 2016) package to perform *post-hoc* pairwise comparisons.

RESULTS

Asexual reproduction

In the first eight days of the experiment, budding rate decreased in each experimental group (Fig. 1). Starting with day 10, the number of buds increased in accordance with the food treatment: budding rate started to increase soonest on the high food level, then on the medium and lastly on the low food levels (Fig. 1; Supplementary Table S1 online).

On account of this dynamic nature of asexual reproduction, here we present only the effect of food amount and perceived density on the last two days of the experiment (results from the preceding days are given in Online Resource 1. We found a significant interaction between food amount and perceived density (LRT: $\chi^2 = 7.709$, df = 2, $P = 0.021$, Fig. 2): high perceived density resulted in reduced budding rate on the low food level (estimate = –0.714, SE = 0.273, $P = 0.027$), but not on the medium (estimate = 0.120, SE = 0.158, $P = 0.896$) or high food levels (estimate = 0.054, SE = 0.140, $P = 0.896$). Animals on medium food had a significantly higher budding rate than the group kept at low food level on both low (estimate = –0.728, SE = 0.196, $P = 0.001$) and high (estimate = –1.562, SE = 0.248, $P < 0.001$) density treatments. There was a marginally significant increase in budding rate at high food level compared to the medium food level at low (estimate = 0.324, SE = 0.152, P = 0.066) and high perceived density (estimate = 0.259, SE = 0.147, $P = 0.078$).

Stress tolerance

The interaction between food amount and perceived density was marginally significant (LRT: $\chi^2 = 4.63$, df = 2, P = 0.099, Fig. 3). Stress tolerance was lower in animals kept in fresh medium on the medium food level (estimate = –2.275, SE = 0.872, $P = 0.027$), but not in the groups kept at low (estimate = –0.979, SE = 0.853, P = 0.502) or high food level (estimate = 0.490, SE = 0.939, $P = 0.602$). High food level resulted in significantly higher stress tolerance compared to the scores measured on medium food in the low density treatment group (estimate = –2.987, SE = 0.924, $P = 0.007$), but not in the high-density group (estimate = –0.222, SE = 0.912, $P = 1$). The difference in stress tolerance between the low and medium food levels was not significant either in the low-density (estimate = –0.568, SE = 0.826, $P = 1$) or the high-density treatments (estimate = 0.727, SE = 0.869, $P = 1$).

Gene expression

Expression level of the housekeeping gene *tuba1* was not influenced by food treatment (F = 0.382, df = 2, $P = 0.689$), density treatment (F = 2.849, df = 1, $P = 0.111$), or the interaction between the two treatments (F = 0.955, df = 2, $P = 0.412$).

The interaction between food availability and perceived density had no significant effect on the transcript level of target genes (Table 2). The expression of two genes (the insulin-like receptor *htk7* and catalase *cat*) was significantly upregulated in the high density treatment groups compared with the low density groups (Table 2, Fig. 4). Food availabil-

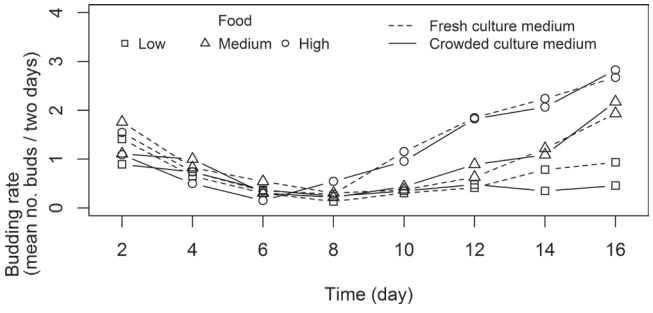

Fig. 1. Number of *Hydra vulgaris* buds (mean ± SE) produced during two days by groups of three hydra kept on different food treatments (low: 10 µl, medium: 30 µl, high level: 90 µl of *Artemia*) and either fresh (low perceived density: 0.6 individuals ml^{-1}) or crowded culture medium (high perceived density: ~ 1.8 individuals ml^{-1}). Error bars (standard error) are omitted for graphical clarity but ranged from 0.054 to 0.264 mean no. buds 2 $days^{-1}$ ($n = 144$ per experimental group)

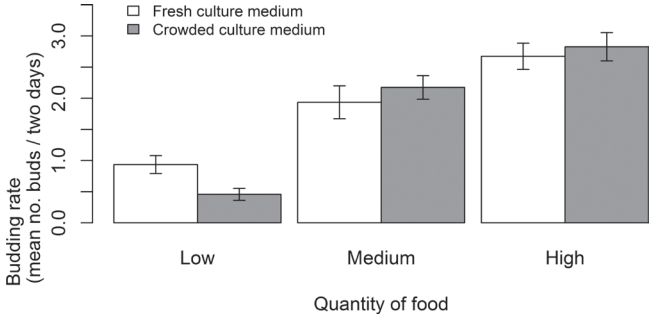

Fig. 2. Budding rate (mean ± SE) per two days on different food levels (low: 10 µl, medium: 30 µl, high level: 90 µl of *Artemia*) of different perceived density groups (low = fresh culture medium: 0.6 individuals ml^{-1}; high = crowded culture medium: ~ 1.8 individuals ml^{-1}) on day 16 of the experiment. Mean number of buds was calculated for three individuals in one well (n= 144 per experimental group).

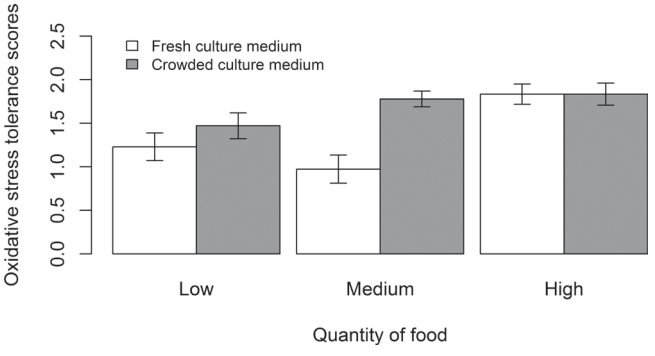

Fig. 3. Oxidative stress tolerance scores (mean ± SE) on day 16 of the experiment of one well containing three hydra kept in fresh (low perceived density: 0.6 individuals ml^{-1}) or crowded culture medium (high perceived density: ~ 1.8 individuals ml^{-1}) and on different food treatments (low: 10 µl, medium: 30 µl, high level: 90 µl of *Artemia*). Stress tolerance was measured by exposing animals to of H_2O_2 based on a morphological scale with 9 levels (n= 207). A score of 0 indicates an entirely disintegrated polyp, while a score of 8 indicates a fully intact animal.

ity alone had no effect on the gene expression patterns of the target genes.

DISCUSSION

In this study we investigated the combined effects of perceived density and food availability on two life history traits (asexual reproduction rate and stress tolerance) and gene expression of IIS pathway members, antioxidants and a heat-shock protein. We found that perceived density influenced both asexual reproduction and stress tolerance but this effect depended on food availability. Perceived density also affected the expression pattern of the insulin-like receptor *htk7* and catalase mRNA levels. However, the expression levels of insulin-like peptide genes and most antioxidant genes did not match the patterns observed in life history traits. We discuss these results in turn below.

First, the effect of the density manipulation on life-history parameters (Fig. 1) was expected based on density-dependent models for population growth (Hassell, 1975). High density is known to cause reduced reproduction in several species (e.g. Ballinger, 1977; Wauters and Lens, 1995), including hydra (Thorp and Barthalmus, 1975). Reducing investment into reproduction may be optimal when population density is high, because the survival chances of offspring produced in such circumstances are low. In high density populations, animals may rather choose to disperse or increase their investment into self-maintenance, to improve their survival prospects until conditions become again favorable for reproduction (both of these are known to occur in hydra; Lomnicki and Slobodkin, 1966; Tökölyi et al., 2014). However, the outcome of this trade-off depends on food availability, because at high resource availability both reproduction and self-maintenance can be maximized simultaneously (van Noordwijk and de Jong, 1986). This prediction was supported by our study, since we found that reproduction of animals kept in crowded culture medium was significantly reduced only in animals exposed to a low food regime, while no such difference was found in animals kept on medium or high food. Similarly, stress tolerance was only affected by density manipulation only under a medium food regime. Hence, stress tolerance was upregulated in animals exposed to high perceived density in accordance with our predictions, but this upregulation did not fully coincide with a downregulation of reproduction. Divergence of budding rate only after day 8 is in agreement with previous studies (Otto and Campbell, 1977), however the highly dynamic nature of hydra responses to food complicate the interpretation of this pattern, since allocation decisions during

Table 2. Likelihood Ratio Tests on the effects of food availability, perceived density, and their interaction on transcript levels of genes encoding insulin-like peptides (*ilp1*, *ilp2*, *ilp3*), the Hydra insulin-like receptor (*htk7*), the heat-shock protein (*hsp70)* and four antioxidant enzymes (*sod*, *cat*, *g6pd*, *gpx*). Significant effects are highlighted in bold.

Gene	Food availability		Perceived density		Food availability* Perceived density	
	F (df = 2)	P	F (df = 1)	P	F (df = 2)	P
ilp1	0.38	0.69	0.23	0.64	0.5	0.62
ilp2	1.37	0.28	2.25	0.15	0.4	0.68
ilp3	0.40	0.67	0.02	0.89	0.45	0.68
htk7	0.26	0.77	**10.33**	**0.01**	2.41	0.13
hsp70	1.57	0.24	0.58	0.46	0.25	0.78
cat	0.49	0.62	**6.42**	**0.02**	0.02	0.98
sod	0.39	0.68	1.66	0.22	0.93	0.42
g6pd	1.19	0.33	0.02	0.88	0.04	0.96
gpx	0.46	0.64	0.93	0.35	0.7	0.52

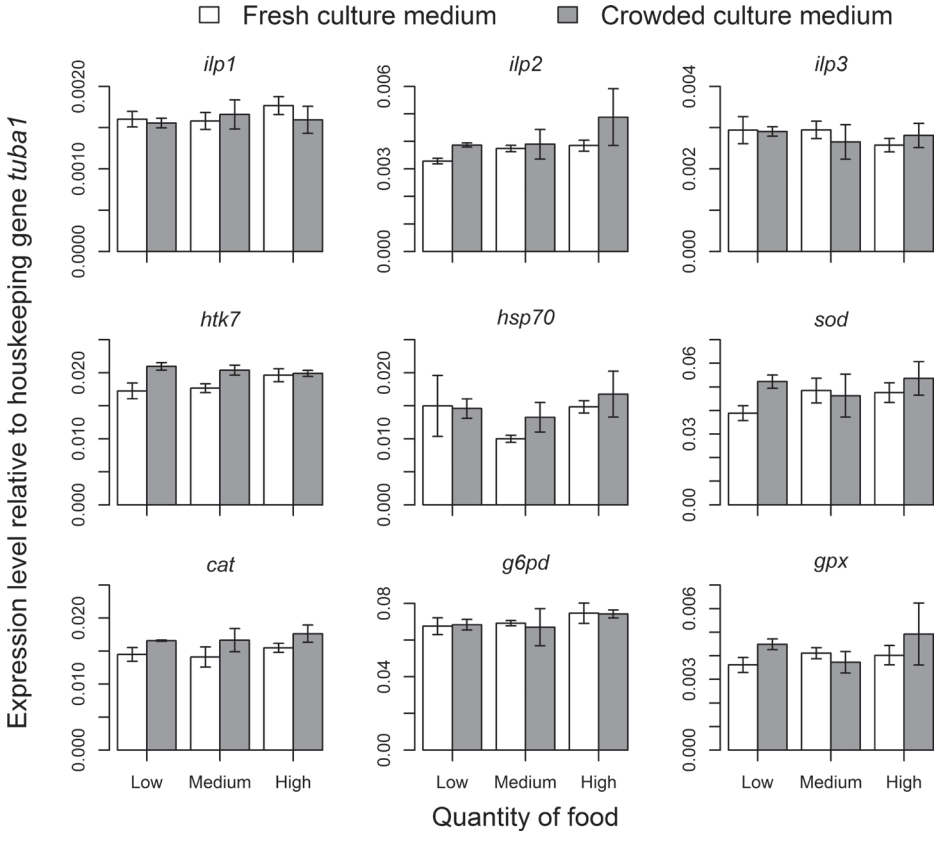

Fig. 4. Expression level of genes (mean ± SE) encoding insulin-like peptides (*ilp1*, *ilp2*, *ilp3*), the insulin-like receptor (*htk7*), the heat-shock protein *hsp70* and four antioxidant enzymes (*sod*, *cat*, *g6pd*, *gpx*) belonging to hydras kept on different food treatments (low: 10 µl, medium: 30 µl, high level: 90 µl of *Artemia*) of different perceived density groups (low: 0.6 individuals ml^{-1} and high: ~ 1.8 individuals ml^{-1}). Transcript levels are expressed relative to *tuba1* expression (n = 12 per experimental group).

the course of the experiment may have influenced stress tolerance at the end of the experiment (e.g. by influencing energy reserves). These results clearly suggest that the two life history traits (asexual reproduction and oxidative stress tolerance) are not directly coupled, but can be regulated differently. Further studies focusing on variation in stress tolerance along the feeding/starvation cycle could help unravel this phenomenon.

Surprisingly, our experimental treatments had no effect on the expression level of insulin-like peptide genes. Hence, differences in budding rate and stress tolerance between food levels are apparently not determined by insulin-like peptides, although we emphasize that mRNA levels might not fully reflect the abundance of these peptides. Insulin-like peptides in general are nutrient-sensitive and mitogenic (Wu and Brown, 2006); unfortunately, very little is known about their function in cnidarians. Treatment of hydra polyps with mammalian insulin has been shown to increase cell proliferation rate (Steele et al., 1996). However, it is unclear whether these three insulin-like peptides (described based on sequence similarity to insulin-like peptides in other organisms; Fujisawa, 2008) function in the same way as in other organisms. Under our experimental conditions, hydras produced some buds at all food levels supporting that cell division occurs even at low feeding regimes (Bosh and David, 1984), which could have influenced expression level of ILP genes, masking the effect of experimental treatments. Furthermore, the IIS pathway may not be the most important pathway regulating reproduction in hydra; for instance, budding is known to be influenced by other signaling molecules as well (e.g. by the hydra *head organizer*; Takano and Sugiyama, 1983), although the relationship of these alternative pathways to nutrient status is unclear. Since budding rates are not linearly dependent on the amount of food received during the feeding / starvation cycle (hydra show a delayed response to changes in food availability), it is likely that multiple pathways are involved. Hence, it is possible that insulin-like peptides are synthesized in the first stage (after receiving food) while other signaling molecules are active at later stages. This is a hypothesis that needs to be tested in the future.

Density treatment did affect expression of the insulin-like receptor *htk7*, with higher expression levels of these receptors observed at high perceived density. Insulin receptors are regulated by a feedback loop in both protostome and deuterostome model organisms. Transcription of *Drosophila* insulin/IGF receptor *dInR* is induced by the absence of insulin while insulin treatment has no effect on *dInR* transcription (Puig et al., 2003). A scenario opposite to this feedback cycle occurs in *Caenorhabditis elegans*, in which overexpression of *ins-1*, a member of *C. elegans* insulin gene family, antagonizes DAF-2 (insulin/IGF receptor) signaling, probably via down-regulation of DAF-2 receptor by a chronic overexpression of its agonist (Pierce et al., 2001). The regulation of insulin receptors in mammals is similar—insulin receptors are downregulated by insulin and upregulated in its absence (Gavin et al., 1974); the latter results in increased physiological preparedness (high receptor levels enabling a quick response to food). Such increased preparedness might allow animals in a high-density population to respond quickly to improving conditions, although other explanations are clearly possible.

Interestingly, mRNA levels for antioxidant enzyme genes and heat-shock protein genes were not higher in experimental groups where stress tolerance scores was higher. This does not necessarily imply that these proteins are not involved in stress tolerance. Indeed, antioxidant genes are known to be upregulated in response to stress in cnidarians (Lesser, 2006) and specifically in hydra (Dash and Phillips, 2012; Woo et al., 2012; Zeeshan et al., 2016). Since we scored stress tolerance after 24 hours of exposure to H_2O_2, antioxidant genes might be upregulated during this time. Hence, differences in stress tolerance among experimental groups may be the consequence of differential ability to upregulate the stress protection system. Our results suggest, however, that hydra do not show a higher level of preparedness in terms of antioxidant enzymes at different food levels or when exposed to crowded culture medium (only catalase was significantly upregulated in the high density groups). On the other hand, since these cellular maintenance genes are targets of the IIS pathway in other animals studied (Vanfleteren, 1993; Honda and Honda, 1999; Clancy et al., 2001; Kops, 2002), an absence of effect in these genes could also reflect the lack of response by the IIS pathway to the experimental conditions.

Overall, our results indicate that asexual reproduction and stress tolerance are influenced by food availability, perceived density, and the interaction between these factors in *H. vulgaris*. IIS appears to play a minor role in determining these responses under our specific experimental conditions. However, given the highly dynamic responses of hydra to food, IIS might be important at some stages of the feeding/starvation cycle. Further studies would benefit by focusing on the temporal changes of insulin-like peptides and insulin-like receptors following feeding, possibly taking other signaling pathways, such as the hydra *head organizer* system into account.

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COMPETING INTERESTS

The authors have no competing interests to declare.

AUTHOR CONTRIBUTIONS

FS, JT, AZL and ZB conceived and designed the experiment. FS, SzP, RR, JB, KL and JT performed the experiments. FS and JT analyzed the data. FS, SzP and JT wrote the manuscript, AZL and ZB provided editorial advice.

SUPPLEMENTARY MATERIALS

Supplementary materials for this article are available online (URL: http://www.bioone.org/doi/suppl/10.2108/zs160171).

Supplementary Table S1. Effects of food treatment, density treatment and their interaction on budding rate during the course of the experiment.

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