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Flexibility in an emergency life-history stage: acute food deprivation prevents sickness behaviour but not the immune response

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The emergency life-history stage (ELHS) can be divided into two subcategories that describe distinct, coordinated responses to disease- or non-disease-related physiological challenges. Whether an individual can simultaneously express aspects of both subcategories when faced with multiple challenges is poorly understood. Emergency life-history theory suggests that disease- and non-disease-related responses are coordinated at the level of the whole organism and therefore cannot be expressed simultaneously. However, the reactive scope and physiological regulatory network models suggest that traits can be independently regulated, allowing for components of both disease- and non-disease-related responses to be simultaneously expressed within a single organism. To test these ideas experimentally, we subjected female zebra finches to food deprivation, an immune challenge, both, or neither, and measured a suite of behavioural and physiological traits involved in the ELHS. We examined whether the trait values expressed by birds experiencing simultaneous challenges resembled trait values of birds experiencing a single challenge or if birds could express a mixture of trait values concurrently. We find that birds can respond to simultaneous challenges by regulating components of the behavioural and immune responses independently of one another. Modularity within these physio-behavioural networks adds additional dimensions to how we evaluate the intensity or quality of an ELHS. Whether modularity provides fitness advantages or costs in nature remains to be determined.

1. Introduction

The flexible adjustment of behaviour and physiology is fundamental to maximizing individual fitness under varying environmental conditions [1]. The ability to respond rapidly to cues that indicate changing environmental conditions is particularly important when animals encounter labile perturbations in their environment, such as intense storms or predation pressure [2]. Under these scenarios, changes in behaviour and physiology allow an individual to maximize survival by suppressing non-essential processes in favour of processes and behaviours that support self-maintenance.

In the late 1990s, Wingfield and others termed the coordinated and stereotyped response to unpredictable events 'the emergency life-history stage' (ELHS) [2,3]. The ELHS is defined by the expression of proactive and facultative changes to animal behaviour and physiology that maintain or restore homeostasis. At the behavioural level, these responses can include the suppression of social behaviours, increased foraging behaviour, or dispersal to new habitat when food availability is compromised [2]. At the physiological level, the ELHS shunts energy towards self-maintenance at the expense of non-essential processes like growth and reproduction, in part through the production of hormones from the glucocorticoid family [2].

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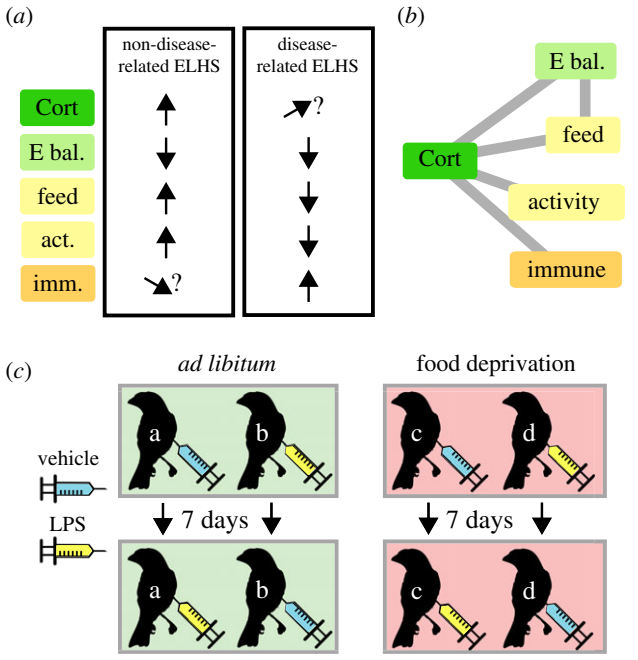

Figure 1. Physiology and behaviour involved in the ELHS responses can be viewed as discrete or part of a more flexible network. (*a*) The ELHS responses have often been presented as two discrete sub-types, non-disease-related and disease-related, which modify the value (up or down) for many traits and their outputs, including corticosterone [Cort] and physiological measures related to energy balance (E bal.). Some traits involved in the ELHS response may be modified in opposite directions depending upon the sub-type, including behavioural traits involved in modulating energy balance (e.g. feeding (feed) and (activity)) and immune function (immune). (*b*) Traits can also be conceptualized to act as nodes within a network where correlations among traits (grey lines) reflect the connectivity of individual nodes. Hub nodes within the network are highly connected to other parts of the network. Cort is hypothesized to act as a hub node within general stress responses and within the ELHS [8,9]. (*c*) We tested whether birds under simultaneous challenge display trait dependence consistent with the traditional ELHS framework (*a*) or with physiological regulatory networks (*b*) by exposing birds to two stressors in a 2×2 design. Groups of birds (a, b, c, d; $N = 8$ per group) were assigned to either *ad libitum* food access or food deprivation, and each individual received injections of vehicle or lipopolysaccharide (LPS), which stimulates an immune response. See Material and methods for further explanation. (Online version in colour.)

The ELHS can be divided into subcategories to describe two classes of whole-organism responses (disease-related and non-disease-related) that fit the broader description of ELHS but differ in key aspects of behaviour and physiology. In response to non-disease-related challenges such as food deprivation, the ELHS includes increased activity, food-seeking, and mobilization of energy stores [2,4,5] (figure 1*a*). By contrast, the disease-related ELHS includes anorexia, decreased activity, and activation of the immune system [6] (figure 1*a*). As currently conceptualized, these categories describe coordinated organismal responses that are, in many ways, mutually exclusive—an animal cannot express both responses simultaneously.

If the ELHS subcategories are biologically incompatible, it raises questions about how an individual should modulate their physiology and behaviour when faced with multiple challenges at the same time, as may occur often in nature. For example, if an animal experiences a sudden decrease in food access simultaneously with infection, which cue(s) does it respond to? In line with the idea that the ELHS subcategories are discrete, Ashley & Wingfield [4,5] hypothesized that the expression of disease- and non-disease-related ELHS must occur sequentially. Ashley & Wingfield [4,5] predicted that individuals should express the disease-related response until they reach a critical energy threshold, at which point animals should switch to the non-disease-related response. Under this model, referred to as the Energy Limitation Hypothesis, the expression of either type of response could be more or less extreme, but the response would be coordinated across the whole organism (i.e. behaviour and physiology should be modified together).

Other popular conceptual models suggest that traits within the physio-behavioural network that comprise an ELHS could respond independently to mixed cues. Such models therefore allow an animal to express aspects of the disease-related and non-disease-related responses simultaneously. The reactive scope model [7] follows along this line by proposing that physiological mediators, including behaviours, can be independently modelled to describe short-term and long-term effects of a challenge on animal resilience. Similarly, physiological regulatory networks [8,9] are based on the idea that traits (nodes) within a system are moderately connected and covary in predictable ways (e.g. figure 1*b*), but that stressors may interrupt connections between traits within a network. Both the underlying network structure and the disruption of key connections among traits could thus allow an individual to express trait values associated with the non-disease-related response at some nodes while simultaneously expressing trait values associated with the disease-related response at other nodes.

There are no studies to date that have quantified physio-behavioural network flexibility within the ELHS per se (but see discussion in [4]). However, it is important to distinguish between these two types of models because the modularity of traits will impact the speed with which animals return to homeostasis during an ELHS and the potential for these responses to evolve independently of one another. The modular nature of the network (or lack thereof) will thus influence the potential cost–benefit ratio of entering the ELHS under a given set of conditions.

To define the network function and flexibility of traits comprising each type of ELHS response, we measured behavioural and physiological trait values in female songbirds responding to challenges that, when presented individually, elicit a strong, coordinated ELHS response: acute food deprivation and immune challenge. We quantified variation in the expression of traits core to the ELHS (figure 1*a*,*b*) in female zebra finches challenged with pseudo-infection (induced by injection with lipopolysaccharide (LPS)), short-term food deprivation, both of these challenges simultaneously or neither (figure 1*c*).

We took three approaches to examine the modularity of traits involved in the ELHS: principal component analysis (PCA), network connectivity within the physiological regulatory network framework, and linear mixed modelling of individual traits.

If ELHS expression is discrete and trait values are coordinated at the level of the whole organism, birds experiencing both challenges will behaviourally and physiologically resemble birds experiencing only food deprivation or only immune challenge. A PCA should thus show that birds experiencing multiple challenges cluster with one of the groups experiencing a single challenge. The physiological

regulatory network of simultaneously challenged birds would resemble the network of birds experiencing a single challenge. Finally, for those traits that respond to both single challenges, linear mixed models should show interactive effects between the two treatments, reflecting that birds experiencing both challenges are only able to express one of the two responses.

If ELHS expression is modular, as predicted by the physiological regulatory network framework, we expected that the PCA would show that birds experiencing multiple challenges would cluster separately from or intermediately to the groups experiencing a single challenge. We followed Martin & Cohen [9] in predicting that, in a covariation network, stress would weaken connections among nodes and thus that additive weakening of connections among nodes would result in the weakest trait connections appearing in simultaneously challenged birds. Finally, we expected that linear mixed models would reflect modularity as either additive or interactive effects of treatments, with effects varying among nodes, reflecting that traits are responding to treatments independent of one another.

2. Material and methods

The experiment was carried out using a breeding colony of zebra finches housed in free-flight aviaries at the Field Station for the Study of Behavior, Ecology, and Reproduction at the University of California, Berkeley. All animal care and procedures were approved by the University of California Office of Laboratory Animal Care and conducted in accordance with local and federal animal welfare laws and policies.

(a) Experimental design

We manipulated food availability (*ad libitum* or food deprived) and immune status (using vehicle or LPS injections) to compare the physio-behavioural response of birds to single and simultaneous challenges (figure 1*c*). Individuals served as their own controls for the immune challenge treatment, receiving LPS or vehicle injections one week apart. The order of injection was split among individuals in each food treatment such that half of the birds received LPS for their first injection and half received vehicle. The experiment was carried out over 2 years, 2017 and 2018, with slight modifications to sample handling detailed below. All treatments were carried out in each year of the experiment. No individuals were used in both years. Sample size for each quadrant totalled 16 individuals. See electronic supplementary material for additional details.

(b) Animal handling and procedures

At the beginning of the experiment, females were uniquely marked with coloured leg bands and permanent marker on the chest. Experiments began at 09.00—birds were caught with butterfly nets, weighed (±0.01 g), injected intramuscularly with 50 µl vehicle (0.9% sterile saline) or LPS (Sigma-Aldrich #L4005, Serotype 055: B5) dissolved in sterile saline, and immediately released back into the colony. The chosen LPS dose (approx. 2 mg kg^{-1}) induces sickness behaviour and an immune response in male zebra finches from this same colony [10–12]. About 4 h following injection, after behavioural observations (see 'Activity' for details), we collected 100 µl of blood from the alar vein immediately following capture such that all blood samples were collected within 10 min of entering the aviary (see 'Blood Collection and Storage'). Birds were weighed a second time prior to release and a third time approximately 24 h after the injection.

For food deprivation treatments, food was collected from inside the aviary 30 min prior to injections (08.30). Water was always available. After 4 h of food deprivation treatment, birds were reweighed to determine change in mass, as described above, and food was replaced.

(c) Activity

Behavioural observations for activity were collected between 11.00 and 13.00 by three trained observers that were blind to the injection type. Observers summed the number of hops and flights and total time spent resting for an individual bird in 5-min bins. After the first observation period, observers switched to a different bird for 5 min, and so on. Each bird was observed for three separate 5-minute bins (once by each observer). Thus, each bird was observed for a total of 15 min.

(d) Feeding behaviour

A video camera was used to record all activity at the food trough from 13.30 to approximately 19.00. Due to video camera memory failure on three of four experimental days in 2018, only video for the 30 min following recapture and mass (approx. 4 h after injection) was used for analyses. A trained observer, blind to treatment, used video footage to record the number of feeding bouts and the duration of each feeding bout for each individual. Chest markings and unique leg band colour combinations were used to identify individuals.

(e) Blood collection and storage

In 2017, blood was collected using heparinized tubes into sterile 500 µl microcentrifuge tubes. Whole blood was held at room temperature until centrifugation (approx. 1 h). In 2018, blood was collected using capillary tubes into Eppendorfs primed with 50 mM EDTA (Invitrogen 15575-020 in sterile saline). About 50 µl of whole blood mixed with EDTA was aliquoted into 1 ml of TRIzol (Invitrogen 15596-026) and held on ice. In both years, blood samples were spun at 1700×*g* for 10 min and plasma was stored in 25 µl aliquots at −80°C.

(f) Total corticosterone

Circulating corticosterone was quantified from all but two birds (missing due to insufficient plasma) using the Arbor Assays ELISA kit (catalogue no. K014). We validated the kit prior to assay with a serial dilution and standard additions using pooled, extracted plasma. Plasma was extracted using a modified diethyl ether extraction [13]. Extraction efficiency was 84 ± 6.5% (mean ± s.d., $N = 8$). All samples were assayed in duplicate, and the average intra-assay coefficient of variation (CV) was 3.48%. Inter-assay CVs were 10.3% and 6.8% for low and high concentration inter-assay controls, respectively.

(g) Plasma lipids (2017 only)

Lipids were extracted from 25 µl of plasma using a modified Folch extraction [14]. Prior to extraction, 0.1 mg of 1-steroyl-rac-glycerol (MAG) was added to all samples as an internal standard. Plasma neutral lipid classes were identified and quantified by thin layer chromatography coupled to flame ionization detection (TLC-FID; [15]).

(h) Blood glucose (2017 only)

Blood glucose was quantified using a commercially available kit (item no. 10009582, Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions. We validated the kit prior to the assay using a serial dilution of pooled serum. Intra-assay variability was 4.1%.

(i) Immune transcript abundance (2018 only)

We used quantitative PCR to examine the differential expression of candidate genes known to be upregulated as part of the immune response to LPS injection [16]. RNA was extracted from whole-blood homogenized in TRIzol using a TRIzol plus column purification approach. Bioline RNA Mini Kit (Bioline, BIO-52073) reagents and consumables were used for the column purification according to the manufacturer's guidelines. One individual yielded too little blood on both days of the experiment for RNA extraction. Primer sequences, concentration used in reactions, annealing temperatures, and efficiencies are provided in electronic supplementary material, table S1. Fold-change in expression was calculated using the Pfaffl method after correcting for assay efficiency [17].

(j) Statistical analyses

Analyses were all run in R v. 3.6.0 [18]. One individual presented an extreme outlier value (greater than 2.5 s.d.s from the group mean) for total corticosterone, and another (different) individual presented an extreme outlier value for mass loss during the experiment. Individuals with outlier values were retained for PCA but excluded from correlation analyses and linear mixed models. Correcting corticosterone for bleed time [19] has no qualitative effect on results (see electronic supplementary material) and can only be done for a subset of the data for which we have exact time to bleed records, so raw corticosterone values were used throughout.

PCA and visualization were carried out using the prcomp() function. Because we did not collect data on physiological immune activation in 1 year, only individuals with complete cases from 2018 were included in the PCA.

Nodes in the physiological network were limited to measures collected around the 4 h timepoint. We considered behavioural traits as part of this network [1,2,20]. Correlations among nodes were examined using Pearson's product–moment correlations; hops, flights, and time spent feeding were transformed using a square root. All correlations with $|r| > 0.50$ were included in plots. We evaluated the strength of covariance among networks by comparing $|r|$ across treatments in two-way ANOVA, following approaches laid out by Merrill & Grindstaff [21]. Networks were plotted using Cytoscape [22].

Effects of challenge (immune, food deprivation, or both) on independent nodes were evaluated using linear mixed models in the lme4 package [23]. All models were fitted using the bobyqa optimizer. A random effect of bird ID nested within the year was included for all measures except those which were only collected in 1 year, in which case only individual ID could be included. For behavioural measures, we included observer set as a fixed effect in the model. A full list of effect sizes and significance are provided in electronic supplementary material, table S2.

3. Results

(a) Principal component analysis

PC1 and PC2 explained 60.9% of the total variance. These principle components were largely able to separate birds across the four clusters corresponding to our full factorial experimental design (figure 2). Birds experiencing both challenges simultaneously clustered in a different part of the plot space than birds experiencing one or the other challenge independently.

(b) Network connectivity and stability

We examined overall topology of the networks across treatment groups (figure 3*a–d*). There were more moderate or strong correlations among network nodes in birds from the *ad libitum*-fed, vehicle-injected (baseline) treatment (figure 3*a*) compared with birds experiencing food deprivation (figure 3*b*) or immune challenge (figure 3*c*), reflecting greater trait covariance across the entire baseline network when compared with other treatments (two-way ANOVA: $F_{1,160} = 11.02$, $p < 0.002$; Tukey's honestly significant differences (HSD): $p < 0.05$ for all comparisons). Birds experiencing both food deprivation and immune challenge had an intermediate number of connections (figure 3*d*), and covariance among traits across the entire network was not significantly different from other treatments (Tukey's HSD: $p > 0.33$ for all comparisons). However, of the 17 correlations appearing in the baseline network, only five appear in any of the 'challenged' networks, and none of these connections were maintained in all networks. In general, there was little consistent overlap among networks. However, some nodes were consistently connected within each network: toll-like receptor 3 (TLR3) and time spent resting (rest) were highly correlated with other nodes across all networks.

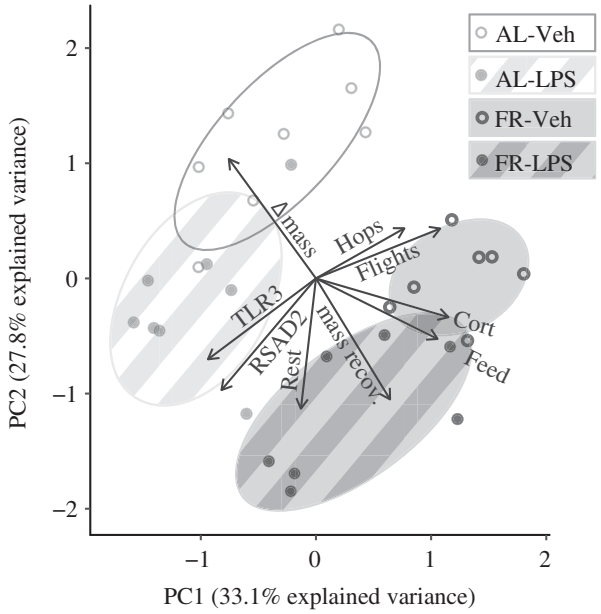

Figure 2. PCA separates birds into four groups corresponding to treatments. *Ad libitum*-fed (AL) birds are shown in pale grey, and food-deprived (FR) birds are shown in dark grey. Open circles show vehicle-injected birds (VEH) and shaded in circles show LPS-injected birds (LPS). Ellipses show confidence ellipse around the group mean for each treatment.

(c) Effects of disease- and non-disease-related challenges on individual traits

We found no interactive effects between challenges on any node in the network (table 1; injection × food, $p > 0.08$ for all models). Six of the nine nodes we examined in this framework responded to only one of the two challenges.

Each behavioural measure responded differently to the various challenges (table 1; electronic supplementary material, figure S1). For example, birds spent more time resting with LPS injection, but less time resting under food deprivation (figure 4*a* and table 1), whereas the amount of time birds spent feeding was only significantly affected by food deprivation (table 1; electronic supplementary material, figure S1).

Food deprivation decreased circulating triglycerides and glucose (table 1; electronic supplementary material, figure S1) and elevated total corticosterone (figure 4*b* and table 1), while LPS injection had no effect on any of these circulating

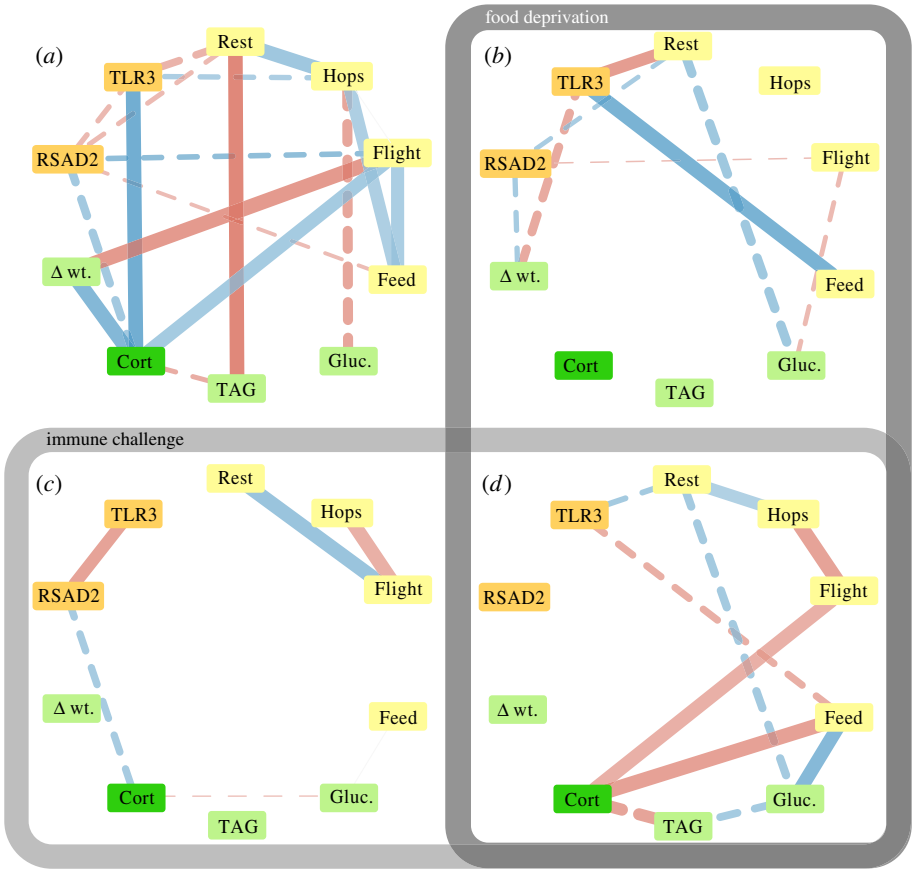

Figure 3. Physiological and behavioural nodes comprising the emergency life-history response show limited correlation and stability across physiological challenges. *Ad libitum*-fed, vehicle-injected animals (*a*) generally display stronger correlations (edges) among nodes than individuals experiencing food deprivation (*b*) or immune challenge (*c*). Animals that experience simultaneous immune challenge and food deprivation (*d*) display unique correlations among nodes. See Material and methods for the explanation of behaviour data (yellow boxes). Other nodes: Gluc., serum glucose; TAG, serum triacylglyerides; Cort, serum corticosterone; Δ wt., change in weight across the experiment; RSAD2 and TLR3, expression of immune-related transcripts in blood. Edges between nodes are shown for correlation coefficients of moderate or greater strength ($|r| > 0.50$). Colour of the edge corresponds to the value of the correlation coefficient (r), ranging from −1 (dark blue) to +1 (dark red). The width of the edge corresponds to p-value, with thicker lines indicating a smaller (more significant) p-value. p-values are uncorrected for multiple comparisons. Edges with a value of $p > 0.05$ are indicated with dashed lines. (Online version in colour.)

factors (table 1). By contrast, expression of both radical *S*-adenosyl methionine domain containing 2 (RSAD2, electronic supplementary material, figure S1) and TLR3 in circulation was elevated in LPS-injected birds (table 1 and figure 4*c*) but was unaffected by food deprivation.

There was a small effect of both food restriction and LPS injection on per cent change in mass during the experiment, with food restriction having nearly twice the effect size of LPS injection on mass loss (table 1; electronic supplementary material, figure S1). Food deprivation resulted in increased mass recovery overnight, and LPS had no effect on mass recovery (table 1; electronic supplementary material, figure S2).

4. Discussion

The two subcategories of the ELHS (disease-related and non-disease-related) require opposing changes to physiological and behavioural traits. While ELHS theory is premised on the idea that animals cannot simultaneously express these two kinds of responses, other theories, including physiological regulatory networks, suggest that traits may be independently regulated to respond to different challenges simultaneously. We predicted that if the two subcategories are mutually exclusive, birds experiencing simultaneous food deprivation and immune challenge should behaviourally and physiologically resemble birds experiencing a single challenge, because individuals must 'choose' a challenge to respond to. If behavioural and physiological traits can be modulated independently, we expected to find that birds experiencing multiple challenges would be able to decouple trait covariation associated with single stressors, allowing them to occupy an intermediate or distinct trait space relative to singly challenged individuals.

(a) Are emergency life-history stage subcategories mutually exclusive across traits?

At the level of the whole organism, we found that birds experiencing simultaneous challenges occupied distinct multidimensional space from individuals experiencing either challenge in isolation (figure 2). We also found that the physiological regulatory network from birds experiencing simultaneous challenges did not resemble the physiological regulatory network found in birds experiencing either challenge in isolation (figure 3). These findings are inconsistent with the hypothesis that ELHS subcategories exist in nature as mutually exclusive responses coordinated at the level of the whole organism (see electronic supplementary material for further discussion on the Energy Limitation Hypothesis). Instead, our results demonstrate that an individual challenged with food deprivation and immune activation can mount both

Table 1. Model parameter significance and effect size estimates. Significant effects are italicized and bolded. All models included year and individual ID as random effects unless otherwise noted. Order of treatment (within individual) was included as a fixed effect in all models (see electronic supplementary material, table S2 for parameter estimates). For behavioural measures (time spent resting, number of hops, and number of flights), the observer set was included as a fixed factor (parameter estimates included in electronic supplementary material, table S2).

outcome	predictors	d.f. (Num, Den)	*F*	*p*	estimate	s.e.
time spent resting (s)	***injection***	***1, 25.94***	***7.85***	***0.01***	***116.70***	***35.87***
	food access	***1, 50.97***	***7.19***	***0.01***	***−107.86***	***62.80***
	inj × food	1, 26.47	3.27	0.08	−91.92	50.81
number of hops	injection	1, 53	1.27	0.26	−27.00	15.25
	food access	***1, 53***	***17.55***	***0.00***	***75.17***	***24.07***
	inj × food	1, 53	1.90	0.17	29.74	21.58
number of flights	***injection***	***1, 25.12***	***13.58***	***0.00***	***6.49***	***1.76***
	food access	***1, 52.25***	***5.35***	***0.03***	***−8.79***	***3.80***
	inj × food	1, 25.586	0.04	0.84	0.37	1.77
time spent feeding[a] (s, afternoon)	injection	1, 28.95	0.05	0.83	0.16	2.14
	food access	***1, 28.79***	***31.56***	***0.00***	***9.75***	***2.36***
	inj × food	1, 29.38	0.01	0.91	0.33	3.04
total corticosterone (ng ml^{-1})	injection	1, 27.74	0.14	0.71	−0.74	2.19
	food access	***1, 30.07***	***24.95***	***0.00***	***10.12***	***2.62***
	inj × food	1, 28.22	0.01	0.91	0.35	3.07
change in mass, 4 h (% initial)	***injection***	***1, 27.79***	***5.39***	***0.03***	***−0.65***	***0.53***
	food access	***1, 29.13***	***5.44***	***0.03***	***−1.07***	***0.67***
	inj × food	1, 28.11	0.30	0.59	−0.41	0.74
glucose[b]	injection	1, 25	0.82	0.39	−0.06	0.07
	food access	***1, 25***	***24.18***	***0.00***	***0.45***	***0.09***
	inj × food	1, 25	0.99	0.34	−0.07	0.07
triglycerides[b] (µmol)	injection	1, 10.56	0.01	0.93	−0.00	0.02
	food access	***1, 11.49***	***16.67***	***0.00***	***0.09***	***0.02***
	inj × food	1, 10.6	0.09	0.77	−0.01	0.02
RSAD[b] (log-fold difference in expression)	***injection***	***1, 11.54***	***151.89***	***0.00***	***1.55***	***0.20***
	food access	1, 15.17	0.55	0.47	−0.52	0.53
	inj × food	1, 12.39	1.18	0.30	0.32	0.29
TLR3[b] (log-fold difference in expression)	***injection***	***1, 8.71***	***87.36***	***0.00***	***0.75***	***0.13***
	food access	1, 11.0	0.32	0.58	−0.19	0.19
	inj × food	1, 9.54	1.06	0.33	0.19	0.19
mass recovered, 24 h (% initial)	injection	1, 57	0.79	0.38	0.66	0.71
	food access	***1, 57***	***41.09***	***0.00***	***3.55***	***0.73***
	inj × food	1, 57	0.18	0.67	−0.43	1.01

[a]Outcome variable transformed using square root for fit; estimates have not been back-transformed.
[b]Year excluded from a random effect because data were only collected in 1 year.

disease-related and non-disease-related ELHS responses by expressing a mixture of trait values.

We also found that individual trait values reflect a summed response to both challenges, rather than any interactive effects (table 1). Of those traits responding to both challenges, two of them (time spent resting and number of flights) had opposing responses of comparable magnitude, suggesting that the trait value expressed by birds experiencing two distinct challenges should be similar to the trait value of birds experiencing neither challenge. Presumably, failing to modify behaviour to meet either challenge would have negative fitness effects. Consistent with this idea, the birds experiencing both challenges lost more mass than birds experiencing either challenge individually. The greater loss of mass suggests a greater immediate cost to the individual. However, the change in mass during the experiment was not consistently related to any other trait under either of the challenges (figure 3; electronic supplementary material, table S3), so we cannot attribute increased cost to any particular component of the response (e.g. activity levels).

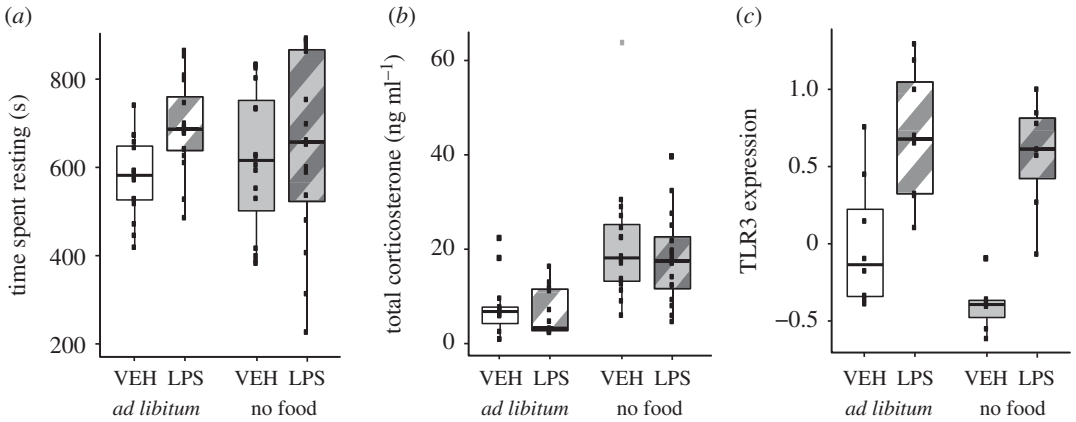

Figure 4. Representative nodes showing effects of an allostatic challenge on the trait value. Boxplots show individual values for behavioural and physiological nodes thought to be involved in the ELHS response, including (*a*) time spent resting, (*b*) total corticosterone in circulation, and (*c*) expression of TLR3. See table 1 for details on significant effects of main treatments (food deprivation and immune challenge). Grey point in (*b*) was excluded from model analyses (see Material and methods). White, empty box: *ad libitum* food access, vehicle injection; white, hashed box: *ad libitum* food access, LPS injection; shaded, empty box: food deprivation, vehicle injection; shaded, hashed box: food deprivation, LPS injection.

(b) Stability and predictability of physiological regulatory networks comprising the emergency life-history stage

Our data support relatively high independence of traits in the physio-behavioural regulatory networks involved in the ELHS. Important to note here is that quantitative approaches for physiological network comparisons are currently limited, so conclusions based on comparisons of network connectivity are largely qualitative. However, consistent with our predictions, we found that covariation strength among nodes in the network decreased when the system was stressed. The connections that were strengthened under stressed conditions tended to appear between traits that were only weakly connected in the baseline network and were inconsistent across stressed states. These patterns reflect a dynamic network.

In addition to predicting decreased connectivity within the network under stress, Martin and Cohen predicted that specific traits should act as highly connected 'hubs' within the network, exhibiting high connectivity to other nodes [8,9]. For example, corticosterone is hypothesized to act as such a highly connected node to coordinate whole-organism responses to stressors [9]. We did not find support for the role of corticosterone as a 'hub' within the network in our manipulation: corticosterone was highly connected within the baseline network, but we found that individual challenges tended to substantially weaken existing correlations corticosterone had with other nodes (see electronic supplementary material, table S3). For example, connections between corticosterone and immune variables were weakened by immune challenge, and all connections between corticosterone and other nodes were eliminated by food deprivation. A potential caveat to these conclusions is that elevated corticosterone, as seen in food-deprived individuals, may have a distinct regulatory role compared with baseline concentrations of corticosterone, and thus, we should expect baseline corticosterone to have different connections than elevated corticosterone [24]. If true, it would be more appropriate to examine corticosterone connectivity in *ad libitum*-fed individuals separate from food-deprived individuals because food deprivation elevates corticosterone (figure 4*c* and table 1). In *ad libitum*-fed birds, where corticosterone is at baseline levels, we find that immune challenge largely disrupts any 'hub' role of corticosterone (figure 3*a* versus *c*). By contrast, under food deprivation, immune challenge *increases* the connectivity of corticosterone (figure 3*b* versus *d*). These opposing effects still do not support a role for corticosterone as a global 'hub' within the ELHS network.

The immune transcript TLR3 exhibits connectivity behaviour that is consistent with it being a 'hub' node within the network: TLR3 connectivity to other traits is retained across all challenges. Time spent resting is also highly connected across networks. These nodes may therefore be better candidates than corticosterone for understanding the topology and stability of physiological regulatory networks in the ELHS.

(c) Whole-organism phenotypes and the emergency life-history stage

Even though birds experiencing simultaneous challenges expressed mixed trait values consistent with both subcategories of the ELHS, food deprivation had a dominant effect relative to an immune challenge across both behavioural and physiological nodes. Unlike immune challenges, food deprivation has a direct, acute effect on energy balance. The activity changes seen during food deprivation are consistent with a shift to constant foraging behaviours. Although these shifts in behaviour occur well before birds experience a negative energy balance [25], the behaviour is directly connected to modifying energy balance. By contrast, the immune response may incur a relatively small cost to songbirds on the scale of hours to days [26–28]. Thus, food deprivation may be more likely to dominate trait values during simultaneous challenges on an acute timescale because the relevance to energy balance is more immediate.

Further complicating these challenge dynamics is the fact that behavioural modulation that occurs during an immune challenge is a motivated behaviour (as opposed to simply energy-dependent) [29]. We found that behavioural nodes are highly coordinated within the behavioural sub-network, but that the sub-network is disconnected from physiological nodes in immune-challenged individuals. This is consistent with the idea that behaviour under an immune challenge is not dependent on current energy balance. By contrast, the

behaviour sub-network displays less connectivity in food-deprived birds, but select behavioural measures are highly correlated with physiological measures. The physiological network under simultaneous challenges, which was associated with increased mass loss, contains more connections between behaviour and physiology sub-networks compared with either challenge on its own. Although behaviour and physiology are undoubtably intimately connected, the specific physiological mechanisms relevant to each type of behaviour are likely important for understanding how nodes within the network are dissociated under stress.

5. Conclusion

We provide experimental evidence that an ELHS can be expressed through modular changes to behavioural and physiological traits. Previously, variation in ELHS expression could be attributed primarily to the intensity of an ELHS response; however, our data demonstrate that variation in the intensity or quality of the response will also reflect modularity in the underlying networks. Quantifying the extent to which physio-behavioural network modularity might provide fitness advantages or confer costs to individuals in the ELHS and across what timescales are critical next steps to understanding the importance of these findings for ELHS expression in natural environments.

Data accessibility. Data are provided in the Electronic Supplementary Materials.

Authors' contributions. The experiment was conceived by K.W. and G.E.B. and carried out by K.W., D.K.E., M.M.A., C.L., and G.E.B. Data acquisition and laboratory analyses were carried out by K.W., C.L., and L.A.T. K.W. analysed data and prepared figures, and K.W. prepared the initial manuscript draft. All authors provided edits and revisions on the manuscript, and all authors approved of the final manuscript.

Competing interests. We declare we have no competing interests.

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