**Supplementary Materials and Methods**

for

Wilsterman, K, MM Alonge, DK Ernst, CA Limber, LA Treidel, and GE Bentley. 2020. Flexibility in the Emergency Life-history Stage: Acute food restriction prevents sickness behavior but not the immune response. *Proc. Royal Soc. B. DOI:* 10.1098/rspb.2020.0842

**Experimental designs:** Experiments were completed between Dec 6-15, 2017 and Dec 1-10, 2018. *Ad libitum* manipulations were carried out on Dec 6th and 13th, 2017 and Dec 1st and 8th in 2018. Food deprivation manipulations were carried out on Dec 8th and 15th 2017 and Dec 3rd and 10th 2018. Individual assignments and treatment orders can be found in the supplemental data file. The mixed-sex colony in which experiments were carried out is exposed to natural changes in day length, with supplemental artificial light on a light/dark cycle of 12:12 (Lights on: 07:00, Lights-off: 19:00). Birds showing signs of reproductive activity (active nesting, incubating, or chick-rearing) were not used in the experiment.

**Feeding behavior:** We were unable to include 4 feeding bouts in analyses because the individual could not be identified from the video footage. These 4 bouts accounted for 1.5% (3 min 44 sec) of the total time spent feeding by all experimental birds across all videos (4 h 12 min 50 sec).

**Total corticosterone extraction and quantification:** Briefly, 5 μL of plasma was mixed into 45 μL of distilled water, and then mixed with 250 μL of diethyl ether for 2 min on an orbital shaker at 1200 rpm. Layers were separated for 5 minutes and then snap frozen in a -80°C freezer. The top layer (diethyl ether containing extracted steroids) was poured into a clean tube and the procedure was repeated. Samples were dried in a SpeedVac and stored at -80°C until assay. Immediately prior to assay, samples were reconstituted in 130 μL of assay buffer. Extraction efficiency was estimated using spike-recovery on a subset of samples. Inter-assay variation was determined using a sample from a baseline individual spiked with standard and a sample from a stressed individual run in duplicate on each plate.

**Plasma lipid extraction and quantification:** All reagents and lipid standards were of the analytical or chromatography grade and purchased from Sigma-Aldrich (St. Louis, MO, USA). Lipids were extracted from plasma using a 2:1:0.9 (v/v/v) mixture of chloroform: methanol: water in glass tubes. Preliminary analyses found there was no detectable MAG present in plasma from zebra finches. Samples were vortexed (1 min.), incubated at RT with gentle shaking (10 min.), and centrifuged (10 min. at 500g) to separate the organic and inorganic layers. The lower organic layer containing both neutral and polar lipids was removed using a glass pasteur pipette and transferred to a clean glass vial. This procedure was repeated, and organic layers were combined. Extracts were dried under N2 gas and reconstituted in 100μl of chloroform. Lipid recovery was between 90-100%. Samples were stored at -20°C for no more than two weeks prior to analysis. Thin layer chromatography coupled to flame ionization detection was carried out by spotting 1.5μl of extracted sample in triplicate onto previously blanked chromorods (Type S5, Silica Shell USA). On the first rod of each set, a standard mix composed of cholesteryl palmitate (CE), stearic acid (FFA), glyceryl tristerate (TAG), cholesterol (Chol), Glyceryl 1,2-distearate (DAG), and 1-steroyl-rac-glycerol (MAG) all at 1mg/ml, was spotted. To separate major classes of neutral lipids present in the plasma, rods were developed in a TLC tank in a solvent system of benzene:chloroform:formic acid (70:30:0.5 v/v/v) until the solvent front reached the 100 mark of the rod holder (~35 minutes). Rods were allowed to dry (5 minutes) before analysis with an Iatroscan MK-6 TLC-FID Analyzer (Shell-USA) (atmospheric air flow rate 2 L/min; hydrogen flow rate 160ml/min; scanning speed 3cm/s). A comparison of retention time of peaks from the standard mix was used to identify lipid components in samples and these components were then quantified using standard curves (0.5-2.5μmol/ml).

**Measures of immune activation**: 750 ng of RNA were reverse transcribed to cDNA in a 20 μL reaction using the BioRad gDNA-clear reverse transcription for qPCR kit (BioRad 1725035). Each sample was assayed in triplicate, 12 μL quantitative PCR reactions containing SYBR green supermix (BioRad 1725271) in 384-well plates on a BioRad CFX 384 Touch Real-Time PCR Detection System with an annealing temperature of 60°C. All primers were validated for specificity (using melt-curves and agarose gel) and efficiency (standard dilution curve) prior to use.

**Statistical Analyses:** For analysis, hops, flights, time spent resting, and time spent feeding were binned within each individual for behavioral analyses. We calculated Δ body mass during the experiments as: , and body mass recovery overnight as: . For linear mixed model analyses, models were fit to raw data except in the case of time spent feeding, which had to be root-transformed to normalize distribution of the model residuals.

The document **Supplementary Materials\_reanalysis** includes details and output for reanalysis of the data where we corrected corticosterone for time to blood collection. There are four tabs that walk through calculation of residual corticosterone and outcomes from each analysis approach.

**Supplementary Discussion**

Can these data offer support or refute the Energy Limitation Hypothesis?

The Energy Limitation Hypothesis (hereafter, ELH) is difficult to test experimentally. Although the ELH predicts discrete categories of emergency life history stage responses, the switch between these two responses is posited to occur at some “change point” linked to an energy threshold. Even if we could reasonably conclude that the energy threshold is similar among individuals (and it seems easy to argue that it might not be), the point in time in which individuals would reach that threshold could vary substantially based on starting condition and intensity of the individual response to the challenge. Said differently, because the rate at which animals will approach the energy threshold can vary among individuals, predictions would need to encompass a range of theoretical conditions any individual may be in. Predictions must also address the fact that components of the non-disease-related emergency life history stage response that occurs after animals reach the aforementioned energy threshold may differ from the non-disease-related emergency life history stage response above that threshold (e.g., mass loss, and other traits that are directly related to energy balance). The wide range of physiological states permitted as consistent with the ELH lead to post-hoc inference that findings are consistent with the hypothesis but largely inhibit *a priori,* experimentally-determined differentiation.

For example, in the case of our experiment, the position of simultaneously-challenged birds anywhere within multivariate space could be consistent with the ELH. If birds fall into the same space as either of the singly-challenged individuals, they are expressing discrete responses but have been sampled at different positions relative to their energy threshold (above or below), and we have no way to otherwise verify where they are relative to this energy threshold. If birds fall in intermediate space, one could argue that this reflects the non-disease-related response that occurs after the energetic threshold has been reached, because measures closely tied to energy balance will differ from birds in singly-challenged treatments whereas behavioral or immune measures will be expressed largely in line with either discrete response. Similar arguments can be made for the physiological networks. Thus, from the *a priori* view, the ELH cannot be distinguished from a “mixed” type response.

However, we believe that our data do provide evidence against the ELH hypothesis when examined post-hoc. Because the ELH is still based on the existence of discrete responses to either challenge type, birds in the simultaneous treatment group should still be expressing a disease-related *or* a non-disease related response at the level of behavior or immune activation. Our behavior data show wide variation in response in the simultaneous challenge treatment (Fig S1A-D), which could be a signal that some birds are in the disease-related emergency life history stage and others are in a non-disease related emergency life history stage, and/or that the “intensity” of each response varies markedly among individuals. However, our immune transcript expression data (Fig S1G,H) show that all individuals in the simultaneous challenge group are universally expressing an immune response and the degree is to the same extent as birds faced with immune challenge alone. Thus, despite variation in behavior that encompasses the range of both emergency life history stage response types, individuals cannot be divided into two groups expressing disease- vs. non-disease responses when we consider that all birds show similar immune activation, regardless of behavioral variation. We can demonstrate this more concretely by repeating our PCA analysis only with trait values that change *either* as part of the immune response (RSAD2 and TLR3) *or* as part of the food deprivation response (Corticosterone, Time spent feeding, and number of hops). By limiting our variables to those that are modified by one or the other response, we should be better able to definitively “sort” individuals from our simultaneous treatment into either type of response. However, we instead find that we still get reasonable separation among the four treatment groups and the majority of simultaneously-challenged birds fall along the diagonal between the treatments, demonstrating that they are expressing a mixture of the disease-related and non-disease-related response.

**Supplementary Legends**

**Table S1 Primers used for quantitative PCR analyses of gene expression on whole blood from female zebra finches.** All reactions were run at an annealing temperature of 60°C. AN: Accession Number.

**Table S2 Complete model output from linear regression analyses.**

**Table S3 Pearson product correlations between nodes in the physiological and behavioral network underlying the Emergency Life History Stage response.**

**Figure S1 All nodes show effects of allostatic challenge on trait value.** Boxplots show individual values for behavioral and physiological nodes thought to be involved in the emergency life history stage response. Behavioral measures included (a) time spent resting, (b) number of hops, (c) number of flights, and (d) time spent feeding. Physiological measures included (e) total corticosterone in circulation, (f) change in mass 4 h following challenge exposure, and expression of two immunity-related transcripts, (g) Radical S-Adenosyl Methionine Domain Containing 2 (RSAD2) and (h) toll-like receptor 3 (TLR3). Significant differences of main treatment effects (food deprivation or immune challenge) are indicated by asterisks (\*) next to the x-axis in each panel. **\*\*\***P < 0.001, **\*\***P < 0.01, **\***P < 0.05, **—** P > 0.05 (See Table 1 for details). Grey points in panels (e) (top) and (f) (bottom) were excluded from model analyses (see 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.

**Figure S2 Food deprivation, but not immune challenge, has sustained effects on body mass.** Birds that experienced food deprivation during the experiment displayed a positive net change in body mass across the experiment, whereas those with *ad libitum* food access tended to display a slightly negative net change in body mass across the experiment. Significant differences of main treatment effects (food deprivation or immune challenge) are indicated by asterisks (\*) next to the x-axis in each panel. **\*\*\***P < 0.001 , **—**P > 0.05 (See Table 1 for details). 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.