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Trade-Offs Underwater: Physiological Plasticity of Rainbow Trout (*Onchorhynchus mykiss*) Confronted by Multiple Stressors

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Abstract: Organisms have evolved mechanisms to partition the available resources between fitness-relevant physiological functions. Organisms possess phenotypic plasticity to acclimate to changing environmental conditions. However, this comes at a cost that can cause negative correlations or "trade-offs", whereby increasing investments in one function lead to decreased investments in another function. The aim of the present study was to investigate the prioritization of resource allocation between growth, pathogen defense, and contaminant response in juvenile rainbow trout (*Oncorhynchus mykiss*) exposed to changes of resource income or expenditure. We performed a multifactorial experiment with three resource-impacting stressors—limited food availability, a parasitic infection, exposure to a vitellogenesis-inducing contaminant—and combinations thereof. Treatment with the individual stressors evoked the expected responses in the respective physiological target systems—body growth, immune system, and hepatic vitellogenin transcription—but we found little evidence for significant negative relations (trade-offs) between the three systems. This also applied to fish exposed to combinations of the stressors. This high phenotypic flexibility of trout in their resource allocation suggests that linear resource allocations as mechanisms of phenotypic plasticity may be too simplistic, but it also may point to a greater capacity of ectothermic than endothermic vertebrates to maintain key physiological processes under competing resource needs due to lower maintenance costs.

Keywords: rainbow trout; resource allocation; food availability; growth; vitellogenesis; immunity; endocrine disruptor; trade-offs

1. Introduction

Phenotypic plasticity is the ability of a given genotype to modulate its phenotype in response to environmental changes, which permits persistence across a range of environmental conditions [1,2]. However, the physiological acclimation or acclimatization of the organism to the environmental change comes at a cost [3–5]. Since the resources of organisms are usually limited, the costs of the stressor response can cause resource competition between fitness-relevant physiological functions, and this may lead to negative correlations or "trade-offs", where increasing investments in one function results in decreased investments in other functions e.g., [6–8]. The challenge that organisms' face is to set priorities on different resource-demanding functions in order to maximize fitness in each environmental situation [7].

Much attention has been paid to resource allocation trade-offs between life history traits, particularly for the reproduction and survival/maintenance components [6,9–11]. For instance, concerning endothermic vertebrates such as mammals and birds, increased cost for reproduction were reported to correlate with reduced investments into immune functions [12,13]. Similarly, pathogen infections can cause a shift of the metabolic priorities of the host to the immune system, redirecting resources away from other functions such as growth [14]. Several studies have proven the importance of hormonal mechanisms underlying trade-offs [15,16]. Moreover, in ectothermic vertebrates such as reptiles and amphibians, negative correlations between the fitness-relevant functions such as reproduction, growth, and immunity have been described [17,18]. In teleost fish, a number of studies have focused on the allocation of resources between growth and locomotor activity, with the latter function influencing foraging success and predator-induced mortality. For example, for strains of rainbow trout (*Oncorhynchus mykiss*) living in environments with different predation risks, Biro et al. [19] proposed the existence of a trade-off between growth and mortality. Likewise, Billerbeck et al. [20] described intraspecific variation in growth among latitudinal populations of the Atlantic silverside (*Menidia menidia)* to be the result of a trade-off between growth and locomotor performance. In contrast, in a common environment experiment with two genetically distinct strains of rainbow trout, Lea et al. [21] did not observe a trade-off between growth and foraging activity. Handy et al. [22] observed that toxicant-exposed rainbow trout covered the costs of detoxification by reducing swimming activity while maintaining body growth. A further question of interest in trade-off studies with fish is the allocation of resources between reproduction and indeterminate growth [23,24].

In this study, we examine physiological trade-offs between fitness-relevant functions as they may result from the acclimatization costs to environmental change. As such, the focus is on within-generation phenotypic plasticity rather than on evolutionary adaptation. The investments into acclimatization to one environmental stressor can lead to negative correlations with other fitness-relevant functions. The physiological trade-offs between two traits are often conceptualized in the so-called Y-allocation model, which illustrates the relative sharing of resource input and the pattern of resource distribution between two systems [10]. In fact, the majority of studies on trade-offs investigate relationships between no more than two fitness-relevant functions [25,26]. However, in their environment, organisms can be exposed to multiple stressors and their cumulative impacts [27–30], raising the question of how this will affect the resource allocation between physiological functions during the acclimatization process.

The aim of the present study was to investigate the prioritization of resource allocation between three physiological functions—body growth, immunity, and vitellogenesis—under exposure to two and three-factorial combinations of natural and anthropogenic stressors. As an experimental model species, we used juvenile rainbow trout. The fish were at the end of their first summer, which is the developmental period in which they manifest high growth rates to increase their chances for overwinter survival [19]. Teleost fish have several physiological peculiarities [31–34]: they are ectothermic, they are able to withstand long periods of food deprivation, they show indeterminate growth throughout their lifetime, and they usually have no brood care. Furthermore, as suggested by Cossins et al. [35], fish species such as rainbow trout from temperate, mid-latitude regions that live in seasonally changing environments may have evolved a high level of phenotypic plasticity to deal with this variation. The specific features of fish may influence their resource needs, and thereby the patterns of resource allocation and physiological trade-offs (cf. [36]). However, fish are not only exposed to predictable stress situations such as seasonal changes, but also to unpredictable stressors such as pathogens and man-made toxicants. A further reason to select fish for the present study on environmentally-induced resource trade-offs is because fish are particularly under pressure by global environmental change, and as a consequence, both their diversity and abundance are declining [37–40]. The environmental stressors that were applied to manipulate the resource status of the trout included: (1) limited food availability, as it occurs in a predictable seasonal way in temperate aquatic habitats, (2) a pathogen infection, as a booster of resource demand, both for the nutrition of the parasite and for the host immune response [41], and

(3) exposure to a man-made contaminant. Contaminants can cause resource expenditures, both for protecting against the toxic activity of the chemical, and for its biotransformation [28,42,43]. Ethinylestradiol (EE2), an anthropogenic stressor, was used as the contaminant, as it is widespread in freshwater ecosystems. In juvenile fish, EE2 induces the synthesis of vitellogenin, which is the precursor of egg yolk, cost-intensive [44–46], and can lead to immune and growth-modulating effects [47,48]. The pathogen that was selected for this study was the myxozoan parasite *Tetracapsuloides bryosalmonae*, which is the causative agent of proliferative kidney disease (PKD) in salmonids [49,50]. Infection intensity of the fish by the parasite reaches maximum values towards autumn, when the fish physiologically prepares for winter. Under the experimental conditions chosen in the present study, the parasite causes a long-term infection and corresponding immune response without causing gross mortalities [51,52]. All three chosen environmental stressors are real world stressors that co-occur in the aquatic habitats of salmonids. In fact, *T. bryosalmonae* and EE2 are considered major factors contributing to the long-term decline of brown trout populations in Swiss rivers [53].

To assess alterations in the allocation patterns under the different scenarios, we measured the changes in organism and organ biomass distribution [48,54] in combination with molecular and physiological parameters. We tested for parameters that are directly linked to the chosen stressors (Table 1).

Table 1. Experimental treatments used in this study, with the life-history trait and the particular parameters used to describe it in each case. EE2: ethinylestradiol, IGF-1: insulin growth factor-1.

Stressor	Life-History Trait	Parameters
Food availability	Growth	Condition factor, IGF-1 expression
Toxicant (EE2) exposure	Induced protein synthesis costs (vitellogenesis)	Hepatic vitellogenesis, liver somatic index
Parasitic infection	Immune parameters	Head kidney somatic index, trunk kidney somatic index, splenosomatic index, expression of immune genes

We hypothesized that the juvenile rainbow trout would prioritize intrinsic growth over the immune defense to the sublethal parasite infection, since rapid growth is decisive for the first winter survival of the fish [55,56]. Furthermore, we expected that the fish would prioritize the immune defense over toxicant defense, as the latter has no physiological function in the juvenile fish: only in mature fish during reproduction.

2. Results

Here, we present a summary of the results from the three physiological systems that may trade off against each other under environmental change: growth, vitellogenesis, and immune system. For a complete outline of the results, as well as additional figures, refer to the Supplementary Materials. Initially, we analyzed the data separately for sex and treatment replicates, although no significant differences were found between neither male and female juvenile trout nor between replicate tanks; the data from both sexes and from replicate tanks were pooled.

2.1. Body Growth

Reduction of the feeding level (LF groups) resulted in a significant decrease of the condition factor (K) in comparison to HF (high feeding) fish (Figure 1a). EE2 exposure had no effect on K. In the parasite-infected fish, the significant difference of K between the LF and HF conditions disappeared, which means that the LF-induced reduction of K was less pronounced in infected fish than in non-infected fish.

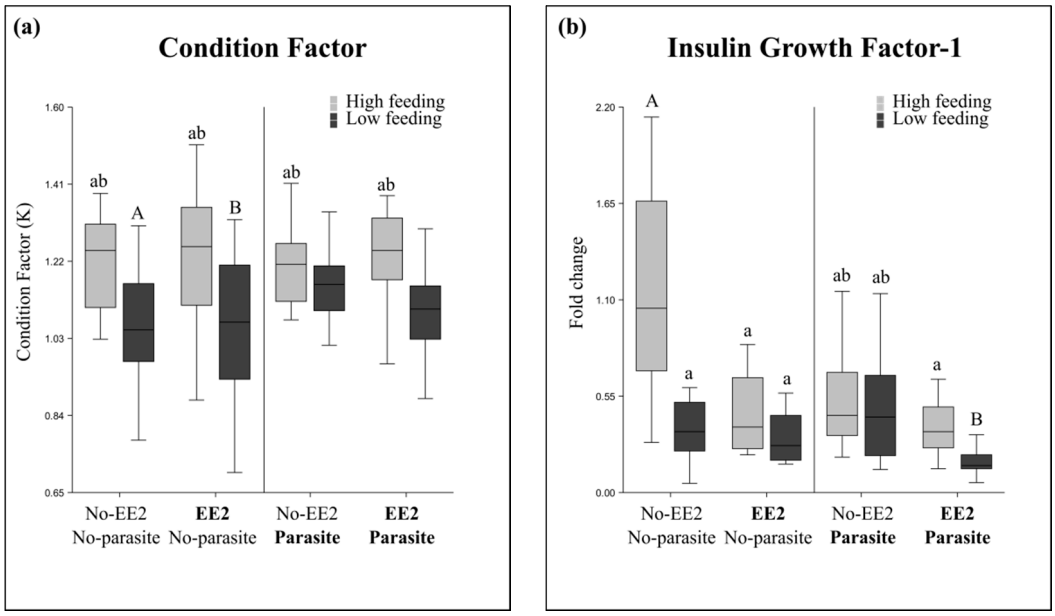


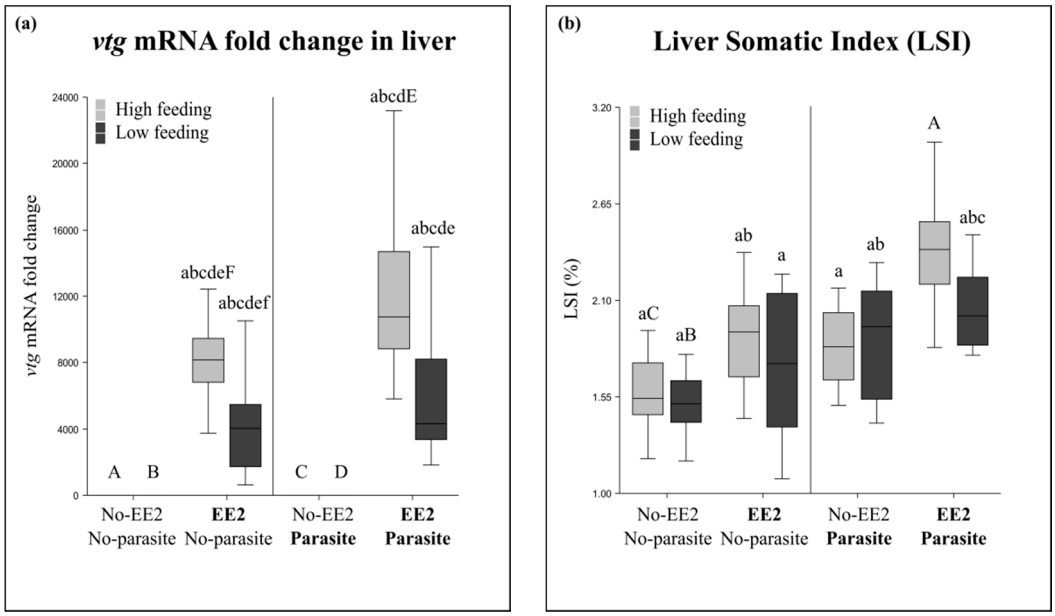
Figure 1. Boxplots of Fulton's condition factor (K) (ANOVA $df = 7$, $p < 0.000$). (**a**) and of insulin growth factor-1 (*igf*-1) mRNA fold change (ANOVA $df = 7$, $p < 0.000$); (**b**) of each treatment group. Post hoc results are available in Supplementary Materials Table S6. Significant differences (at $p < 0.05$) exist between boxplots with capital and lower letter superscripts (for instance "A" indicates a significant difference relative to "a", and "B" indicates a significant difference relative to "b").

The specific growth rate (SGR) of the animals over the 118-day duration of the experiment (Supplementary Materials Figure S1a) was reduced when the feeding level was reduced. Neither EE2 nor parasite infection had a clear effect on the SGR, but the combination of infection, low feeding level, and EE2 (LFEP group) led to the lowest SGR among all of the groups.

As a molecular marker related to somatic growth, we measured the hepatic mRNA expression of the insulin growth factor-1 (*igf*-1) gene. In comparison with the HF group, all of the treatments led to a significant reduction of hepatic *igf*-1 mRNA levels. This finding contrasts the observation on the K values. For instance, while EE2 exposure did not significantly change the condition factor of HF fish, it significantly decreased the *igf*-1 mRNA levels of fish from this treatment. Interestingly, the combination of all stressors—low feeding, EE2 exposure, and parasite infection—led to significantly lower *igf*-1 expression compared to all of the other groups, correlating with the SGR data.

2.2. Contaminant-Induced Vitellogenesis

Fish not exposed to EE2 expressed virtually no endogenous hepatic vitellogenin (*vtg*) transcripts. However, when exposed to the estrogenic contaminant, EE2, *vtg* mRNA levels were significantly increased. This took place under both LF and HF conditions (Figure 2a), but the amplitude of *vtg* mRNA elevation was lower in the LF group than in the HF group. Parasitic infection had no significant effect on the increase of *vtg* mRNA levels.



(**a**) (**b**)

Figure 2. Boxplots of the hepatic vitellogenin (*vtg*) mRNA fold change (*ANOVA df* = 7, $p < 0.000$). (**a**) and the liver somatic indexes (*ANOVA df* = 7, $p < 0.000$); (**b**) of each treatment group. Post hoc results are available in Supplementary Materials Table S6. Significant differences (at $p < 0.05$) are indicated with capital and lower letter superscripts. For instance, "A" indicates a significant difference relative to "a", "B" indicates a significant difference relative to "b", and so on ("C" versus "c", "D" versus "d", "E" versus e", and "F" versus "f" in each graphic).

As *vtg* synthesis takes place in the liver, an increase of *vtg* mRNA could increase the relative liver weight (liver somatic index, LSI). However, we did not observe a significant increase of the LSI in EE2-exposed juvenile rainbow trout, despite a tendency for liver enlargement (Figure 2b). Parasite infection alone had no significant effect on the LSI, but additional EE2 exposure tended to increase the LSI, which was a trend that became significant for the HFEP (high feeding, EE2, and parasite) group.

2.3. Immune System and Infection Status

The immune system response under the various treatments was assessed by measuring the organ somatic indices of two key immune organs in fish—the head kidney (HKSI) and spleen (SSI)—as well as the mRNA transcript levels of immune genes involved in the host response of rainbow trout to the infection by *T. bryosalmonae*. The selection of the genes was based on the findings reported by Bailey et al. [51] and Gorgoglione et al. [57] on the rainbow trout immune response to *T. bryosalmonae* infection.

The low feeding level did not result—against expectation—in reduced immune organ indices, but led to a non-significant trend for increased HKSI and SSI (Figure 3a,b). Parasitic infection had a pronounced effect on the immune organ indices, significantly increasing the HKSI and SSI, whereas the EE2 remained without effect on the immune organ indices.

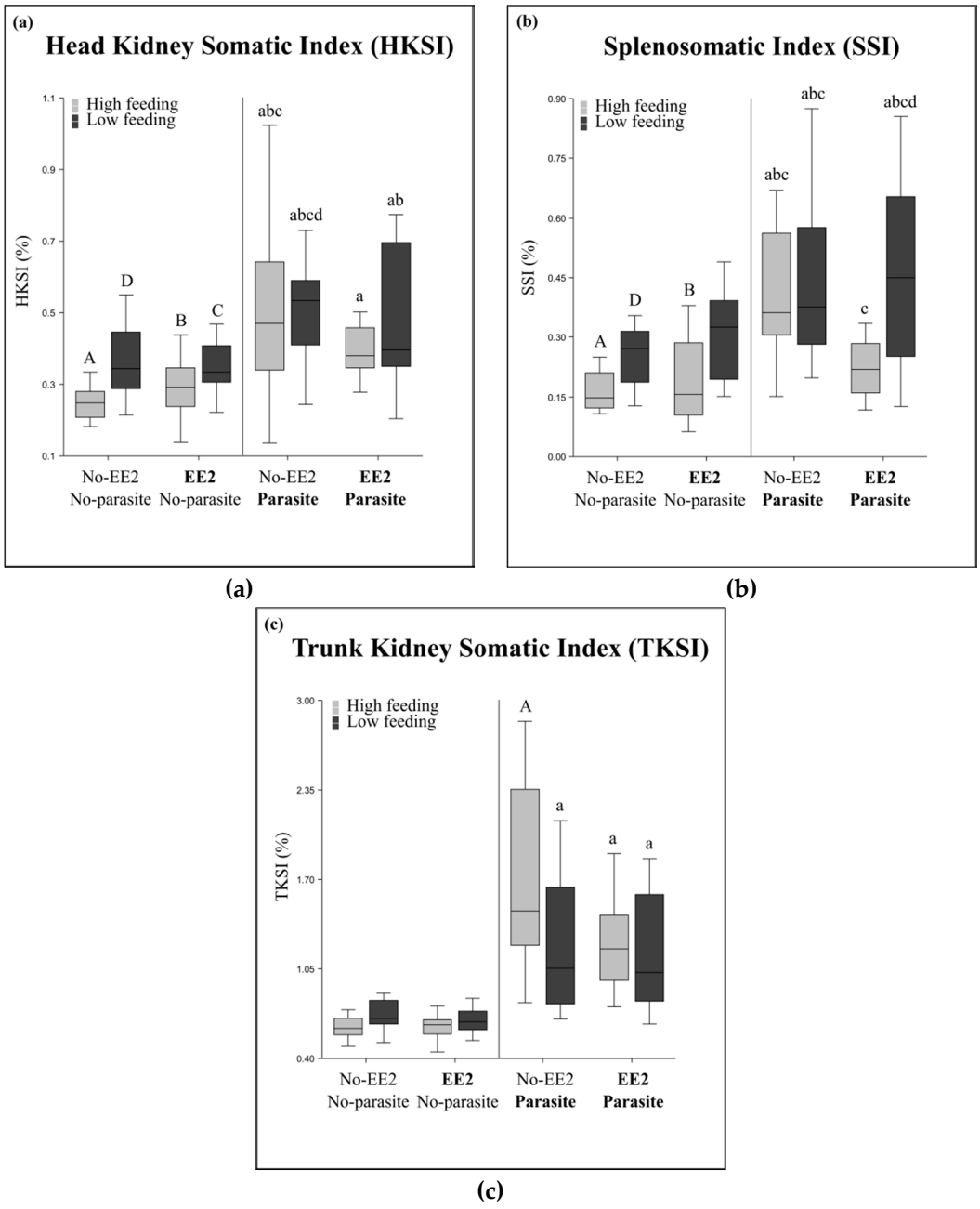


Figure 3. Boxplots of head kidney somatic indexes (*ANOVA df* = *7*, $p < 0.000$) (**a**), spleenosomatic indexes (*ANOVA df* = *7*, $p < 0.000$), (**b**) and trunk kidney somatic indexes (TKSIs) (*ANOVA df* = *7*, $p < 0.000$); (**c**) of each treatment group. Post hoc results are available in Supplementary Materials Table S6. Significant differences (at $p < 0.05$) are indicated between boxplots with capital and lower letter superscripts. For instance, "A" indicates a significant difference relative to "a", "B" indicates a significant difference relative to "b", and so on ("C" versus "c", "D" versus "d", "E" versus e" and "F" versus "f" in each graphic). Additionally, all of the TKSI values from the infected groups were significantly higher than those from all of the uninfected groups.

The main target organ of the parasite is the trunk kidney, which typically experiences an enlargement due to parasite proliferation and the ensuing host immunopathology [58]. Thus, the trunk kidney somatic index (TKSI) can be taken as a proxy for parasitic intensity [51]. In fish infected with *T. bryosalmonae*, the TKSI significantly increased (Figure 3c). Importantly, fish that received a lower food level (LF) or were exposed to EE2 showed a significantly lower elevation of the TKSI values than the HF fish.

Parasitic infection was the main factor altering the expression of the immune genes *blimp*-1, *IgM-sec*, *IL-10*, and *NKEF* in the head kidney, which were significantly increased in infected fish compared to non-infected fish (Figure 4). Low feeding levels resulted in a weaker response of the immune genes compared to their response under high feeding conditions. The estrogenic exposure had no impact on the immune gene expression levels.

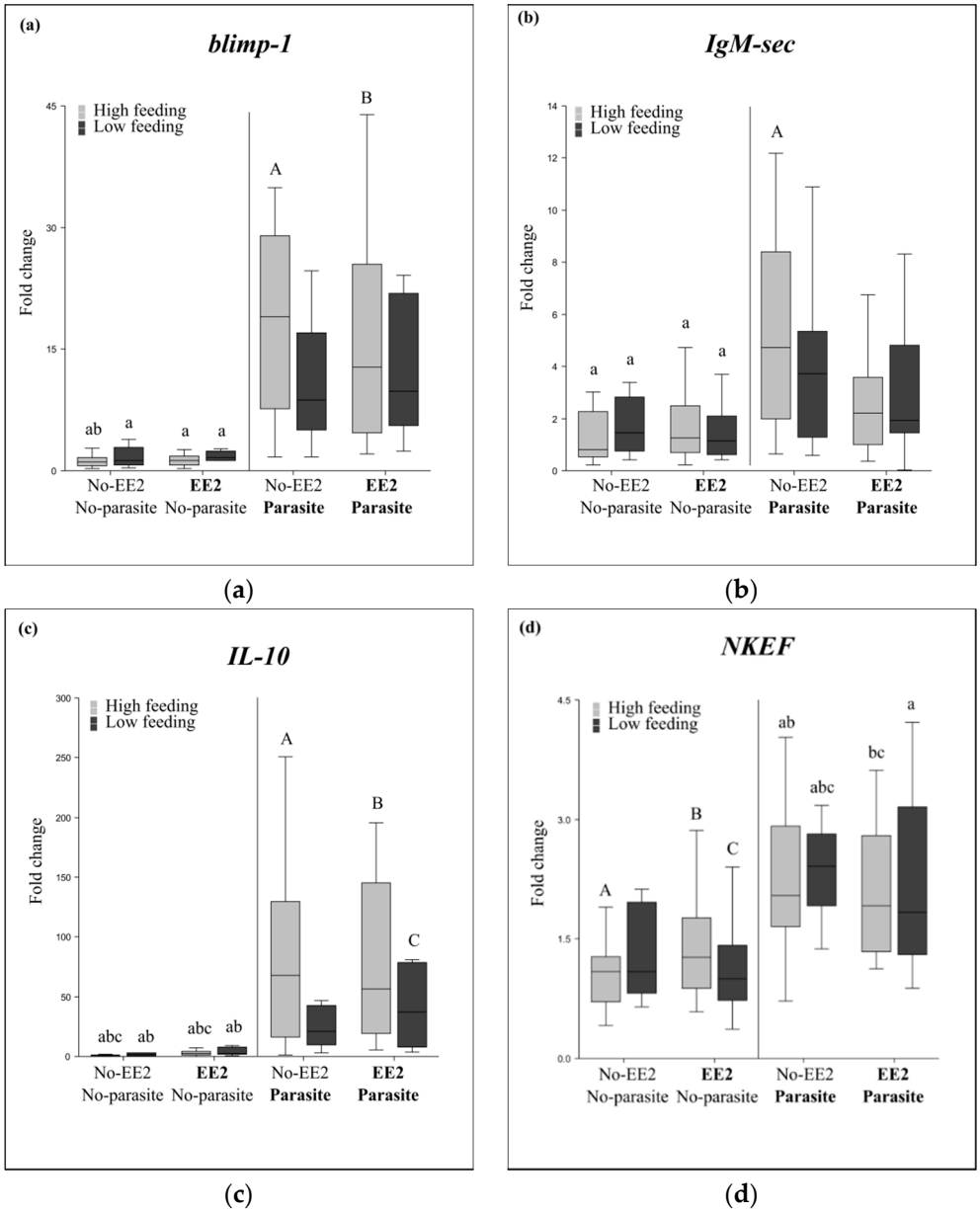


Figure 4. Boxplots of fold changes of *blimp-1* (*ANOVA df = 7, p* < 0.000). (**a**), *IgM-sec* (*ANOVA df = 7, p < 0.000*); (**b**), *IL-10* (*ANOVA df = 7, p* < 0.000); (**c**) and *NKEF (ANOVA df = 7, p < 0.000)*; (**d**) mRNA levels measured in the head kidney for all of the treatment groups. Post hoc results are available in Supplementary Materials Table S6. Significant differences (at $p < 0.05$) are indicated between boxplots with capital and lower letter superscripts. For instance, "A" indicates a significant difference relative to "a", "B" indicates a significant difference relative to "b", and "C" versus "c").

2.4. Analysis of Treatment Effects

The multifactorial study design enabled us to identify the effects of all of the treatments, individually or in combination, on the experimental parameters. A factorial ANOVA was performed for all of the parameters measured and all of the treatments (Table 2). EE2 treatment significantly affected the parameters related to vitellogenin synthesis as well as hepatic *igf*-1

transcript levels and two immune parameters (TKSI, *IgM-sec*). The parasite infection had a significant impact on all of the immune parameters, the LSI and, again, hepatic *igf*-1 expression. Reduced food availability led to significant changes of all the growth and vitellogenin-related parameters, but had no significant effects on the immune parameters. Treatment of the fish with combinations of stressors evoked less significant effects than the single stressor treatments (Table 2).

Table 2. Factorial ANOVA of the effects of the single stressor and combined stressor treatments on all of the experimental parameters.

	EE2 Exposure			Parasitic Infection			EE2 Exposure x Parasitic Infection			Feeding Level			EE2 Exposure x Feeding Level			Parasitic Infection x Feeding level			EE2 Exposure x Parasitic Infection x Feeding Level		
PARAMETER	**DF**	**F-Ratio**	**Prob Level**	**DF**	**F-Ratio**	**Prob Level**	**DF**	**F-Ratio**	**Prob Level**	**DF**	**F-Ratio**	**Prob Level**	**DF**	**F-Ratio**	**Prob Level**	**DF**	**F-Ratio**	**Prob Level**	**DF**	**F-Ratio**	**Prob Level**
Condition factor (K)	1	0.12	0.73	1	1.18	0.28	1	0.29	0.59	1	35.53	0.00 *	1	1.55	0.22	1	1.79	0.18	1	0.87	0.35
HKSI (%)	1	1.18	0.28	1	47.22	0.00 *	1	2.97	0.09	1	7.05	0.01*	1	0.01	0.91	1	0.5	0.48	1	2.37	0.13
TKSI (%)	1	4.71	0.03*	1	113.63	0.00 *	1	3.54	0.06	1	2.98	0.08	1	2.01	0.16	1	8.36	0.00*	1	3.57	0.06
LSI (%)	1	30.26	0.00*	1	39.05	0.00 *	1	0.65	0.42	1	8.14	0.01 *	1	6.44	0.01 *	1	0.18	0.67	1	3.49	0.06
SSI (%)	1	1.09	0.29	1	32.46	0.00 *	1	3.61	0.06	1	21.81	0.00 *	1	3.55	0.06	1	0	0.95	1	3.61	0.06
Liver *IGF-1* (-ddCt)	1	33.87	0.00*	1	10.37	0.00*	1	0.31	0.57	1	41.69	0.00 *	1	0.17	0.68	1	1.03	0.31	1	12.91	0.00*
Liver *vtg* (-ddCt)	1	4794.73	0.00*	1	2.07	0.15	1	1.35	0.24	1	10.73	0.00 *	1	4.66	0.03*	1	0.33	0.56	1	0.04	0.83
NKEF(-ddCt)	1	0.68	0.41	1	51.73	0.00 *	1	0.32	0.57	1	0.49	0.48	1	4.15	0.04 *	1	0.01	0.92	1	1.11	0.29
Blimp-1(-ddCt)	1	0.18	0.67	1	171.88	0.00 *	1	1.18	0.28	1	0.23	0.62	1	0.67	0.41	1	4.92	0.02*	1	0.86	0.35
IgM-sec (-ddCt)	1	4.15	0.04*	1	17.34	0.00 *	1	3.02	0.08	1	0.04	0.83	1	0.2	0.65	1	0.71	0.40	1	1.83	0.17
IL-10 (-ddCt)	1	3.72	0.06	1	169.91	0.00 *	1	0.5	0.48	1	0.01	0.93	1	0.03	0.86	1	9.49	0.00 *	1	0.15	0.69

* Significant effects of the treatment on the given parameter ($p < 0.05$). HKSI: head kidney somatic index; LSI: liver somatic index; SSI: splenosomatic index; TKSI: trunk kidney somatic index; DF: degrees of freedom.

2.5. Negative Correlations between the Parameters Related to Growth, Immunity, and Contaminant-Induced Vitellogenesis

Negative correlations between the parameters of growth, contaminant exposure, and pathogen defense would be suggestive of phenotypic trade-offs between these functions. To assess the presence of such trade-offs, we analyzed the correlations between all of the measured parameters from all of the treatments (Table 3). When analyzing all of the individuals together, significant negative correlations were found primarily for hepatic *IGF*-1 expression: over all of the treatments, this parameter showed negative correlations with HKSI, SSI, hepatic *vtg* expression, *NKEF*, *Blimp*-1, and *IL*-10 head kidney expression (Figure 5).

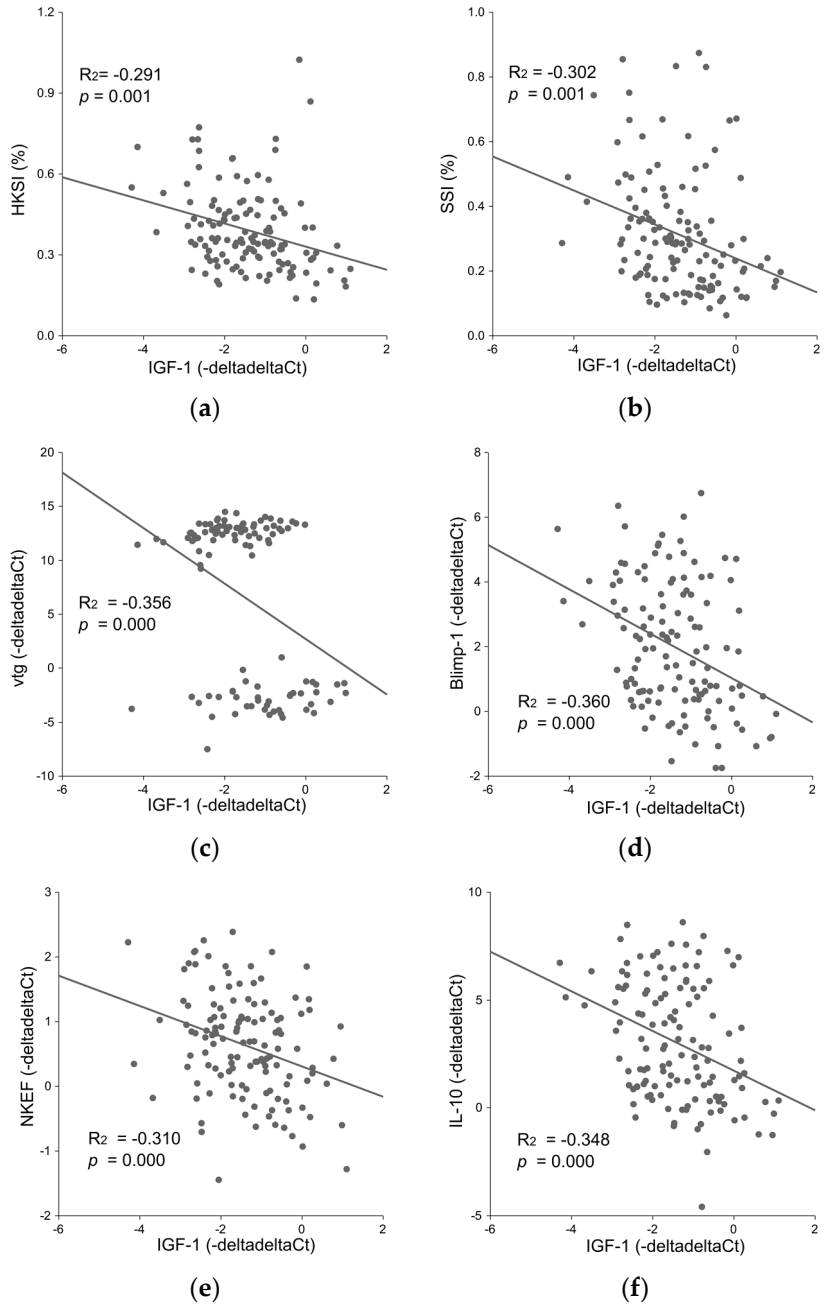


Figure 5. Correlations between *IGF*-1 mRNA expression and (**a**) HKSI; (**b**) SSI; (**c**) *vtg* mRNA expression; (**d**) *Blimp*-1 mRNA expression; (**e**) *NKEF* mRNA expression; and (**f**) *IL*-10 mRNA expression.

Table 3. Pearson Correlations Section (pairwise deletion) between all of the parameters presented.

		Condition Factor (K)	HKSI (%)	TKSI (%)	LSI (%)	SSI (%)	Liver *IGF-1* mRNA (-ddCt)	Liver *vtg* mRNA (-ddCt)	*NKEF* mRNA (-ddCt)	*Blimp-1* mRNA (-ddCt)	*IgM-sec* mRNA (-ddCt)	*IL-10* mRNA (-ddCt)
Condition Factor (K)	*Correlation*	1.00	−0.10	0.06	0.387 *	−0.235 *	0.458 *	−0.02	−0.17	−0.05	0.05	−0.03
	Significance	0.00	0.25	0.54	0.00	0.01	0.00	0.87	0.06	0.60	0.59	0.72
	n	128.00	128.00	128.00	127.00	128.00	128.00	106.00	128.00	128.00	128.00	128.00
HKSI (%)	*Correlation*	−0.10	1.00	0.702 *	0.274 *	0.580 *	−0.291 *	0.08	0.530 *	0.693 *	0.463 *	0.658 *
	Significance	0.25	0.00	0.00	0.00	0.00	0.00	0.44	0.00	0.00	0.00	0.00
	n	128.00	128.00	128.00	127.00	128.00	128.00	106.00	128.00	128.00	128.00	128.00
TKSI (%)	*Correlation*	0.06	0.702 *	1.00	0.363 *	0.569 *	−0.11	−0.02	0.538 *	0.792 *	0.483 *	0.744 *
	Significance	0.54	0.00	0.00	0.00	0.00	0.22	0.83	0.00	0.00	0.00	0.00
	n	128.00	128.00	128.00	127.00	128.00	128.00	106.00	128.00	128.00	128.00	128.00
LSI (%)	*Correlation*	0.387 *	0.274 *	0.363 *	1.00	0.10	−0.10	0.444 *	0.229 *	0.358 *	0.198 *	0.408 *
	Significance	0.00	0.00	0.00	0.00	0.24	0.29	0.00	0.01	0.00	0.03	0.00
	n	127.00	127.00	127.00	127.00	127.00	127.00	106.00	127.00	127.00	127.00	127.00
SSI (%)	*Correlation*	−0.235 *	0.580 *	0.569 *	0.10	1.00	−0.302 *	0.00	0.390 *	0.542 *	0.339 *	0.516 *
	Significance	0.01	0.00	0.00	0.24	0.00	0.00	0.99	0.00	0.00	0.00	0.00
	n	128.00	128.00	128.00	127.00	128.00	128.00	106.00	128.00	128.00	128.00	128.00
Liver *IGF-1* mRNA (-ddCt)	*Correlation*	0.458 *	−0.291 *	−0.11	−0.10	−0.302 *	1.00	−0.356 *	−0.310 *	−0.360 *	−0.10	−0.348 *
	Significance	0.00	0.00	0.22	0.29	0.00	0.00	0.00	0.00	0.00	0.27	0.00
	n	128.00	128.00	128.00	127.00	128.00	128.00	106.00	128.00	128.00	128.00	128.00
Liver *vtg* mRNA (-ddCt)	*Correlation*	−0.02	0.08	−0.02	0.444 *	0.00	−0.356 *	1.00	−0.05	0.10	−0.09	0.18
	Significance	0.87	0.44	0.83	0.00	0.99	0.00	0.00	0.59	0.31	0.37	0.07
	n	106.00	106.00	106.00	106.00	106.00	106.00	106.00	106.00	106.00	106.00	106.00
***NKEF* mRNA (-ddCt)**	*Correlation*	−0.17	0.530 *	0.538 *	0.229 *	0.390 *	−0.310 *	−0.05	1.00	0.656 *	0.458 *	0.641 *
	Significance	0.06	0.00	0.00	0.01	0.00	0.00	0.59	0.00	0.00	0.00	0.00
	n	128.00	128.00	128.00	127.00	128.00	128.00	106.00	128.00	128.00	128.00	128.00
***Blimp-1* mRNA (-ddCt)**	*Correlation*	−0.05	0.693 *	0.792 *	0.358 *	0.542 *	−0.360 *	0.10	0.656 *	1.00	0.615 *	0.888 *
	Significance	0.60	0.00	0.00	0.00	0.00	0.00	0.31	0.00	0.00	0.00	0.00
	n	128.00	128.00	128.00	127.00	128.00	128.00	106.00	128.00	128.00	128.00	128.00
***IgM-sec* mRNA (-ddCt)**	*Correlation*	0.05	0.463 *	0.483 *	0.20	0.339 *	−0.10	−0.09	0.458 *	0.615 *	1.00	0.500 *
	Significance	0.59	0.00	0.00	0.03	0.00	0.27	0.37	0.00	0.00	0.00	0.00
	n	128.00	128.00	128.00	127.00	128.00	128. 128	106.00	128.00	128.00	128.00	128.00
***IL-10* mRNA (-ddCt)**	*Correlation*	−0.03	0.66	0.74	0.41	0.52	22120.35	0.18	0.641*	0.888*	0.500*	1.00
	Significance	0.72	0.00	0.00	0.00	0.00	0.00	0.07	0.00	0.00	0.00	0.00
	n	128.00	128.00	128.00	127.00	128.00	128.00	106.00	128.00	128.00	128.00	128.00

* Significant effects of the treatment on the given parameter ($p < 0.05$).

The correlations between all of the parameters were also analysed individually for each treatment group, and the results are presented in Supplementary Materials Table S2.

2.6. Principal Component Analysis

Multivariate statistics were used to unravel patterns in the physiological resource allocation between the three physiological functions under the different treatments. For this, three principal component analyses (PCA) were performed on the variables describing (1) body growth, (2) contaminant defense (hepatic vitellogenin synthesis), and (3) parasite defense (immune parameters).

For all three PCAs, only the first component (PC1) had an eigenvalues higher than one. Therefore, only the first component were analyzed further. The first component of the PCA performed on body growth parameters explained 71.84% of the variation. Meanwhile, the first component of the PCA on the vitellogenesis parameters explained 79.33% of the variation, and the first component of the PCA on the immune system accounted for the 65.49% of the variation.

In a next step, a Pearson correlation analysis was performed on the PCA coefficients of the first components (PC1) to identify the negative correlations between the parameters of the three physiological functions.

A significant negative correlation was observed between vitellogenesis and immune parameters, suggesting the existence of a trade-off between these two physiological functions (Table 4). Interestingly, between immune system and body growth, there appeared to be a significant positive correlation.

Table 4. Pearson correlations (pairwise deletion) between the first component of the principal component analysis (PCA) scores for each life history trait, body growth, vitellogenesis, immune system, and disease status.

		Body Growth PC1	Vitellogenesis PC1	Immune System and Disease Status PC1
Body growth PC1	*Correlation*	1		
	Significance	0		
	n	128		
Vitellogenesis PC1	*Correlation*	−0.01609	1	
	Significance	0.869965	0	
	n	106	106	
Immune system PC1	*Correlation*	0.231636 *	−0.232971 *	1
	Significance	0.008517	0.016247	0
	n	128	106	128

* Significant correlations ($p < 0.05$).

3. Discussion

This study investigated how juvenile rainbow trout alter the resource allocation between three physiological systems—body growth, immune system, and vitellogenesis—when exposed to a single environmental stressor, or a combination of environmental stressors. Three stressors, to which fish are exposed to in their environment, were selected: (i) reduced food availability as it occurs seasonally in temperate latitudes during winter and leads to a decrease of resource income, (ii) infection by a parasite, which is an unpredictable event that causes enhanced resource expenditures to manage the pathogen, and (iii) sublethal exposure to a contaminant, which again is an unpredictable event that causes increased resource investments. We expected that the juvenile fish would prioritize intrinsic growth—as it is of relevance for overwinter survival—over immune defense against the parasite infection, and the immune defense over contaminant-induced vitellogenesis, as the latter has not yet a physiological function in the juvenile fish. In contrast, our data show that immune defenses were prioritized over growth, and there was little evidence for general trade-offs between the traits.

3.1. Effects of Reduced Food Availability

Reduced food availability did lower investment in growth-related parameters (decrease of condition factor, specific growth rate, and hepatic *igf*-1 mRNA expression) and EE2-induced vitellogenesis (decrease of LSI and hepatic *vtg* mRNA levels). Surprisingly, the immune investment as indicated from the immune organ somatic indices, HKSI and SSI, was significantly enhanced at the reduced feeding level (LF). This finding is in contrast to what would be expected from a resource allocation trade-off perspective per se, but it is in agreement with the findings from other studies, which reported that the immune system gets preference in situations of reduced resource income. For example, the small-scaled pacu fish *Piaractus mesopotamicus* was shown to increase plasma lysozyme activity during starvation periods [59]. Similarly, endothermic vertebrates such as birds or mammals have been reported to enhance their immune functions under limited food access [8,60–62]. Given that immune responses are costly [7,11,63,64], the prioritization of the immune parameters under reduced resource income is surprising at a first glance. However, as discussed by Rauw [14], during periods of reduced food availability, maintenance functions, including immunity may be given priority over growth, as they promote the survival of the organism. In this context, the findings of Buehler et al. [8] are of interest; these authors found that a bird species maintained constitutive immune functions such as microbial killing ability, which was maintained under food limitation, although other, more costly aspects of the immune defense such as the acute phase reaction were suppressed.

3.2. Effects of Parasite Infection

Parasite infection led to an activation of the immune system, as evident from the increase of SSI, TKSI, and HKSI, together with the significant upregulation of the immune genes b*limp*-1, *IgM-sec, NKE,F* and *IL*-10. These genes are prominently involved in the immune response of rainbow trout against the parasite *T. bryosalmonae* [51,52,57]. Since a parasite infection is costly, both with respect to the body resources consumed by the parasite [64] and with respect to the activation of the host immune system, the infection should correlate with a decreased investment in other activities such as growth or contaminant-induced vitellogenesis. This was indeed shown for hepatic *igf*-1 mRNA expression in parasite-infected fish, but we observed neither a significant negative correlation between most of the immune parameters and the condition factor nor between TKSI (proxy for infection intensity) and vitellogenesis. Vice versa, a reduced resource income due to low feeding levels or increased resource expenditures due to contaminant-induced vitellogenesis did not correlate with a reduction of immune investment. Overall, except for the *igf*-1-response, the data from this study provide no evidence that an increased investment in the immune response correlates with a reduced investment in growth or contaminant response. As already mentioned above, this finding is in agreement with what is to be expected from a resource allocation trade-off perspective.

3.3. Effects of the Contaminant Exposure

The third stressor that was used to manipulate the resource status of juvenile rainbow trout was the exposure to the estrogen-active contaminant EE2. This compound activates—via the estrogen receptor—the hepatic synthesis of vitellogenin, which is cost-intensive [45], and thus should lead to trade-offs with growth and/or immunity. Although EE2 exposure did increase *vtg* mRNA and LSI, no negative correlation was observed with the other physiological system, except for the downregulation of hepatic *igf*-1 mRNA. Obviously, at the molecular level, the fish "sensed" the resource conflict, but this did not translate into more apical effects.

3.4. Effects of the Combinations of Stressors

Increasing the pressure on the resource status of the juvenile trout by exposing them to combinations of stressors did not change the response of trout compared to fish exposed to only one stressor. For example, infection of the trout with the parasite led to a significant increase of

immune organ indices and immune gene expression. This investment to fight the pathogen was maintained under the combined influence of reduced resource income (lower food availability) and increased resource expenditure (increased vitellogenesis). Remarkably, the only treatment that led to a partial attenuation of the immune response toward the pathogen was the combination of the high (not the low!) feeding level with EE2 exposure.

Past studies demonstrated numerous examples of trade-offs between the immune response and other resource-demanding activities [11,18,28,65–67]. However, experimental observations do not always support such simple relationships [68–70]. As a case example, a study on house sparrows (*Passer domesticus*) by King and Swanson [71] described that the activation of the immune system incurred energetic costs, but still did not trade off against thermogenic performance. Likewise, Williams et al. [72] found for European starlings (*Sturnus vulgaris*) that enhanced immune function did not trade off with the reproductive performance, and concluded that a resource allocation model assuming straightforward negative correlations between physiological traits may be over-simplistic. Our findings further corroborate that trade-offs are not linear relationships between two physiological systems, but that the phenotypic plasticity of organisms can involve more complex relationships between physiological functions. As formulated by Garland [73], "*expected trade-offs based on biological principles may not be found, as nature has more degrees of freedom than assumed by simple conceptualizations*".

This leads to the question: how can organisms maintain certain functions despite the increasing pressure on their resources? One possible explanation could be that trade-offs appear only when the resource investments are substantial [14,66]. While immunity is usually considered to be cost-intensive, few quantitative data are available [32,67], so that the costs for the immune system activation may be too low to result in a trade-off with other functions, although there exist reports on trade-offs between immune system activation and other physiological functions [28,74]. Another factor to be considered is the overall resource balance of the organisms [75]. An example of this is the difference between capital and income breeders [76], with the former taking the resources for breeding from the stored resources, while the latter have to generate the investment from the actual income. The juvenile fish that were used in the present study may have lower stored energy resources as they aim to maximize their growth rates, but since we have not measured the energy content of the fish, we cannot definitely exclude this possibility. Another important mechanism that may support the flexibility in trade-offs is the ability of organisms to prioritize resource allocations. An excellent literature example on the importance of priority rules for a flexible resource allocation is provided by the study by Jokela and Mutikainen [2], who demonstrated that freshwater clams under limited resource availability prioritize maintenance (i.e., immunity) over reproduction and reproduction over growth. This also occurred in the present study, as the immune system was prioritized over other functions: when the fish were subjected to reduced food availability, they strongly reduced their investments into growth and vitellogenesis, but they increased the relative size of the immune organs. Similarly, when the fish were infected with the parasite, they maintained their immune response, irrespective of whether their resource income was reduced or whether they had additional expenditures for vitellogenesis.

Finally, physiological trade-offs may differ between ectothermic and endothermic vertebrates. Ectotherms may be better than endotherms at maintaining the functionality of key physiological processes under competing resource demands, as it happened in this study, due to the lower thermoregulation costs. This is corroborated by findings from other ectothermic species such as the garden snake (*Thamnophis elegans*), which showed remarkable plasticity in maintaining vital functions when exposed to multiple stressors [36]. Similarly, in the leopard frog (*Lithobathes sphenocephalus*), reduced food availability and pathogen infection did not result in trade-offs with growth [77]. Furthermore, in the pacu fish *Piaractus mesopotamicus,* no trade-off between food availability and immunity was observed [59]. Conceivably, ectothermic vertebrates, due to their lower maintenance costs, possess a high degree of physiological plasticity, with resource conflicts becoming manifest only under rather strong or extreme resource limitations [73,75]. It would be

rewarding to test these hypotheses in future comparative studies, since the results of the present study provide evidence for this.

4. Materials and Methods

4.1. Experimental Design

This study included three experimental treatments: (1) different feeding levels, (2) infection with a parasite, and (3) exposure to an environmental contaminant. The experimental groups and exposure timeline are summarized in Figure 6. To study possible interactions between the three experimental factors, we applied a sequential exposure scenario: after quarantine (see below) and a 14-day acclimation period to the experimental tanks, the fish were classified either in the high-feeding level (HF) or the low-feeding level (LF). This was done for 14 days to allow the metabolism to acclimate to the dietary regime. Thereafter, fish were exposed to 17α-ethinylestradiol (EE2) to induce vitellogenesis (HFE, LFE), while the remaining tanks further received uncontaminated control water (HF, LF). This treatment was done for another 14 days to induce an estrogenic physiological condition in the EE2-exposed fish, as it was evident from a significant upregulation of hepatic *vtg* mRNA in the treated groups (data not shown). Finally, at day 28 after initiating the experiment, exposure with the myxozoan parasite, *Tetracapsuloides byrosalmonae*, was done in the four treatments (HFP, LFP, HFEP, and LFEP) to trigger an immune response (for details on the infection procedure, see Section 4.6). Infection success was confirmed by detecting the presence of the parasite in the fish trunk kidney via qPCR. Afterwards, all of the treatment groups were reared until day 118 after the start of the experiment.

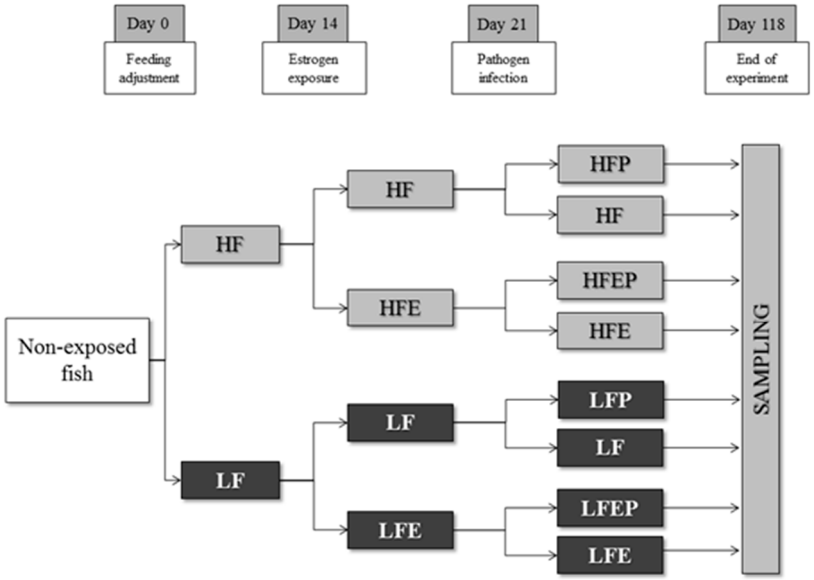


Figure 6. Experimental treatments and their temporal sequence, leading to the following groups: **HF:** High feeding level, no EE2 exposure, no pathogen infection; **LF:** Low feeding level, no EE2 exposure, no pathogen infection; **HFE:** High feeding level, EE2 exposure, no pathogen infection; **LFE:** Low feeding level, EE2 exposure, no pathogen infection; **HFP:** High feeding level, no EE2 exposure, pathogen infection; **LFP:** Low feeding level, no EE2 exposure, pathogen infection; **HFEP:** High feeding level, EE2 exposure, pathogen infection; **LFEP:** Low feeding level, EE2 exposure, pathogen infection.

4.2. Animal Care

The animals that were used for the experiment were six-month-old rainbow trout (*Oncorhynchus mykiss*) weighing approximately 10 grams. Fish were obtained from the cantonal fish farm of Rodi, Switzerland (Piscicoltura Cantonale Rodi, 6772 Rodi, Switzerland). Upon arrival, fish were screened for the presence of pathogens, including *T. bryosalmonae*, and they were found to be free of infectious agents.

After four weeks of quarantine at the facilities of the Centre for Fish and Wildlife Health, Bern, Switzerland, animals were transferred into 35-L flow-through glass tanks. The flow rate was set at 60 L of tap water/h, with a 12-h light 12-h dark-cycle (12L:12D). Average water temperature was 12.9 (±1.22) °C. Feeding was carried out daily using Hokovit Trout Start feed (Hokovit, Switzerland). Every tank contained 122 fish at the beginning of the experiment, meaning a rearing density of 35 grams/liter. Fish were continuously sampled, and at the end of the experiment, each tank contained 52 animals, and there was a density of 81 g/L in the tanks receiving the high feeding level, and 41 g/L in the low-feeding tanks. Fish numbers and densities are given in Supplementary Materials Table S3.

All of the procedures were carried out according to the Swiss legislation for animal experimentation guidelines, license number BE102/16.

4.3. Feeding Regimes

Once fish were distributed to the tanks, tanks were randomly selected as HF or LF groups. HF groups were fed at a ratio of 1.5% of body weight per day, which corresponds to a commonly used feeding level in aquaculture for this size of fish. LF corresponded to a daily feeding of 0.75%. Pellet size was adjusted every second week to fish size, and the amount of food given per tank was adjusted to the weight per fish and the number of fish per tank.

4.4. Ethinylestradiol (EE2) Exposure

The estrogen exposure was performed with 17α-ethinylestradiol (EE2, purity ≥98%) (E4876-10G, Sigma-Aldrich, Buchs, Switzerland). The synthetic hormone was dissolved in molecular biology purpose ethanol (purity ≥ 99.8%) (Merck, Darmstadt, Germany), and then diluted in the appropriate volume of water in a 20-L glass beaker with a magnetic rotor on the bottom to mix the solution. The solution was then pumped into the fish tanks through inert tubes (Flow Tubing, Pharamed, 4.0 mm ID, Gilson AG, Mettmenstetten, Switzerland) using peristaltic pumps (Minipuls3®, Gilson AG, Mettmenstetten, Switzerland). The flow rate was adjusted to achieve the targeted EE2 concentration (nominal 10 ng/L) in the fish tanks. The actual water inflow was controlled using Flow Rotameters (Rota Yokogawa, Wehr, Germany). The concentration of EE2 in the tanks was analyzed throughout the duration of the experiment (see Section 4.5).

4.5. Analytical Determination of the EE2 Concentrations in the Tank Water

The nominal water concentration of EE2 in the fish tanks was 10 ng/L. To verify this concentration, water samples were analyzed monthly. Water samples were collected using SPE C18 filter cartridges (Grogg Chemie, Stettlen, Switzerland) attached to a vacuum flask. Before use, the filter cartridge was cleaned and pre-conditioned with five ml of methanol, followed by 10 mL of distilled water. Then, 250 mL of the fish tank water was passed through the cartridge at a speed of 20 mL/min. The cartridge was then washed with five ml of water, dried for one minute, washed with five mL of hexane (20 mL/min), and EE2 was eluted using five ml of dichloromethane at a rate of three mL/min. The solvent was evaporated, the dry residue was resolved in 100 µl of 100% methanol, and adjusted to one milliliter with pure water. The EE2 concentration in the sample was measured using a competitive EE2 Elisa Kit (Ecologiena®, Fukuoka, Japan) following the users instruction manual. Briefly, the conjugate solution and the standards (or the samples) were mixed in an uncoated microplate. The solution was then transferred into an EE2 monoclonal antibody-coated microplate, incubated for 60 minutes, and washed two times. Dye solution was added, and

after 30 minutes of incubation, the reaction was stopped, and the color intensity was measured in an EnSpire plate reader (PerkinElmer, Waltham, MA, USA) at the 450-nm wavelength. The EE2 concentration was on average 5.5 ng/L.

4.6. Parasite Exposure

For our pathogenic infection, we selected the myxozoan parasite *Tetracapsuloides bryosalmonae,* the causative agent of proliferative kidney disease (PKD), which is a disease of wild and farmed salmonid populations. The infection was performed in a controlled manner as described by Bailey et al. [51] and Strepparava et al. [78]. In short, infected bryozoans, which are the invertebrate host of *T. bryosalmonae,* were collected from a natural river in Switzerland that is known to be endemic for the parasite, and analyzed for its presence. To release the parasite from the bryozoans into the water, the bryozoans were disrupted and diluted in water. Water flow was stopped in all of the tanks (independent of the treatment), and homogenous aliquots of this solution were added to the fish tanks of the parasite-exposed groups for a fixed time window (one hour) to infect the fish. After this hour, water flow-through was started again, and the remaining infective parasite spores were washed out. The infection was repeated for four days consecutively.

The concentration of parasites was selected, based on our previous studies with PKD [51,52,78], at a level that is sufficient to infect the fish, but does not cause fish mortalities. The exposure procedure that was used in this study was based on a limited pathogen exposure window of one hour over four days; thereafter, the fish were kept in parasite-free water. This procedure avoids the continuous reinfection of the fish, but ensures a defined starting point for infection prevalence, parasite proliferation, and the host immune response [51,52,78].

4.7. Fish Sampling

The results presented here are taken from the sampling performed at day 118 after initiating the experiment. Day 118 was selected because at that stage, the parasite intensity in the fish host had reached its plateau. From each duplicate, eight fish were sampled (n = 16 per treatment). Each duplicate was held in a different tank.

Fish were euthanized using 150 mg/L of buffered MS222® (3-aminobenzoic acid ethyl ester, MS222®, Argent Chemical Laboratories, Washington, WA, United States). The length and weight of each fish were recorded with a precision of millimeters in length and centigrams in the weight. The animals were dissected, and the weights of the gonads, liver, spleen, head kidney, and trunk kidney were recorded. The fish were sexed by a histological examination of the gonads.

Fulton's condition factor (K) was calculated with the formula:

$$K = W \times L^{-3} \times 100$$

where W is weight in grams, and L is total length in cm.

The specific growth rate (SGR) represents the speed at which the animals gained the weight. It was calculated by dividing the gain of weight through the period through the number of days, using the following formula:

$$SGR = (\text{final mean weight} - \text{initial mean weight})/\text{total number of days}$$

The somatic index of each organ was calculated by dividing the organ weight by the total weight of the animal and presenting it as a percentage.

For RT-qPCR and qPCR measurements, the head kidney, half of the trunk kidney (lengthwise), and a piece of the liver were placed into individual tubes containing 1500 µl of TRI Reagent® (Sigma-Aldrich, Buchs, Switzerland) and a five-mm stainless steel bead (Qiagen, Hombrechtikon, Switzerland). The samples were then homogenized using a tissue lyser (Qiagen, Hombrechtikon, Switzerland) and stored at −80 °C.

4.8. Gene Expression of Immune System Related Genes and IGF-1

4.8.1. RNA Isolation

After thawing the samples, one mL of the homogenized tissue in TRI Reagent® was added to 200 µl of chloroform in a new tube and centrifuged at 16,000 rcf for 15 minutes at 4 °C. The top layer was transferred into a new tube with 500 µl of isopropanol and centrifuged again. The final pellet was washed with 70% ethanol twice. The RNA was resuspended in RNase/DNase-free water. The concentration of RNA was quantified by Nanodrop.

4.8.2. cDNA Synthesis and RT-qPCR Analysis

cDNA was synthesized using the GoScript™ Reverse Transcription Mix (Random Primer, Promega, Dübendorf, Switzerland) following the manufacturer's instructions. For each sample, one ug of RNA was used. qRT-qPCR was performed in 96-well Fast Optical reaction plates (Thermofisher Scientific, Reinach, Switzerland) following the SYBR-green type protocol by adding 6.25 µl GoTag qPCR MasterMix (2×) (Promega, Dübendorf, Switzerland), 0.625 µl from each primer (10×) (Table 5), 2.5 µl of molecular water, and 2.5 µl of the sample per well, working on duplicates. The reaction was performed using an Applied Biosystem 7500 FAST analyzer (Applied Biosystems, Foster City, CA, USA). The reaction mixture was incubated for five minutes at 95 °C, followed by 45 cycles of amplification. Each cycle had three seconds of denaturation at 95 °C, annealing, and elongation at 60 °C for 30 seconds. Data was evaluated according the 2^{-ddCt} method [79], and Elongation Factor-1α was used as a reference gene.

Table 5. Rainbow trout oligonucleotides used for RT-qPCR assays.

Gene	*Acc. Number*	*FWD Primer (5-3)*	*REV Primer (5-3)*	*BP*	*References*
Ef-1α	AF498320	TGCCCCTGGACACA GAGATT	CCCACACCACCAGC AACAA	90	[80]
IgM sec	X65261	TACAAGAGGGAGA CCGGAGGAGT	CTTCCTGATTGAATC TGGCTAGTGGT	221	[57]
Blimp-1	NA XM 014204594.1	AGCTGTCCAACCTC AAGGTCC	TTGCGGCACACCTGG GCATTC	NA	[81]
NKEF	NM_001124277.1	TGCCGAGGAGTTTA GGAAGA	AATCTTCATGGCACC CAGAC	62	[82]
IL-10	NM_001245099.1	CTGCTGCTCCTTCG TAGAGG	CTCGTCATTAGCCTC GTAGTAGTCTC	94	[51]
IGF-1	not available	TGGACACGCTGCA GTTTGTGTGT	CACTCGTCCACAATA CCACGGT	120	[83]
vtg	XM_021599796	CGCAGTTAAATGTA GCATGGTC	TTGAGCTCTGTGGTG CAATC		

4.9. Statistics

The differences between treatment groups were tested using one-way analysis of variance (ANOVA) and significant differences were revealed with the Tukey–Kramer multiple comparison *post hoc* test. Data failing normality was tested statistically using the non-parametric Kruskal–Wallis ANOVA on ranks, followed by the non-parametric Dunn's multiple comparison test to reveal the differences (for each parameter, see Supplementary Materials Table S4). Differences were considered statistically significant if $p \leq 0.05$. In order to analyze potential interaction effects, a factorial ANOVA was performed. The negative correlations between all of the parameters were analyzed with the Pearson's correlation coefficient. To get insights about general trade-offs, a principal component analysis was performed on the parameters forming the groups of growth, vitellogenesis, and immunity and disease status. The coefficients of each first component of the PCA were used to analyze for negative correlations, and correlations were tested with Pearson's Test. All of the statistical tests and graphical presentations were performed with NCSS 10 Statistical Software (2015 NCSS, LLC. Kaysville, UT, USA, ncss.com/software/ncss).

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: Specific Growth Rate measured during the experimental period, along with length and weight. Table S2: Pearson correlations section (pairwise deletion) between all measured parameters. Table S3: Monthly mean values of maintenance conditions through the experiment in terms of fish numbers and densities, from the start of the experiment in July until the final sampling time point in January. Table S4: ANOVA results for comparison between groups of all parameters. Table S5: Statistical analysis applied for each parameter. Significance was given when $P \leq 0.05$. Tabe S6. P-values and Z-values of the multiple comparison tests for each parameter between all of the groups. Significant values are shown in bold.

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