Heritable and environmental influences on innate immunity across three populations of threespine stickleback (*Gasterosteus aculeatus*)

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Keywords: threespine stickleback, innate immunity, ecoimmunology, microbial environment, 16S DNA, granulocytes, ROS.

Abstract

Environmental variation plays a key role in immune development and function; factors such as pathogen exposure history, seasonality, and resource availability all affect an individual's immune phenotype. However, the relative contributions of heritable and non-heritable factors remain unclear for most immune phenotypes. We used populations of threespine stickleback (*Gasterosteus aculeatus*) with heritable differences in immune function to investigate the relationship between immunity, genetic divergence, and the environment. Fish were raised in tanks with different flow rates (continuous or intermittent) that harbored differing microbial communities. After long-term acclimation to one tank environment, a subset of adult fish were moved to the alternate flow regime and allowed to acclimate for eighteen weeks. We then measured the effects of starting environment, transfer between environments, and final environment across several immune parameters. Both population and treatment significantly affected immune function. Sticklebacks from a population previously found to display the highest heritable levels of innate immunity displayed the highest oxidative burst capacity (ROS) regardless of tank environment. However, all fish in intermittent flow tanks (both resident and transfers) tended to have higher ROS production, more granulocytes, and greater spleen mass than in continuous flow. Variation in liver mass was mainly driven by population effects. Together, this work demonstrates that a simple change in water flow dynamics can shift aquarium microbial conditions, creating opportunities to explore immune flexibility. Perhaps more importantly, we also highlight the need for further research examining how naturally evolved genetic differences and environmental factors individually and jointly influence the magnitude and direction of immune responses.

Introduction

Differential immune responses facilitate an organism's ability to maintain high fitness in the face of changing pathogen risk. While some elements of immunity are highly innate, others are influenced primarily by either environment or by genotype-by-environment interactions. Ecoimmunology has traditionally focused on environmental factors such as seasonality, resource availability, and exposure to pathogens (1). This is largely due to difficulty in identifying heritable differences in wild organisms, which is further complicated when environmental influences vary across the life of an organism. For example, many species have a critical window where immune system development is sensitive to external inputs. In these cases, the adult immune function is contingent on early life exposures (2,3). In contrast, other aspects of immunity display plastic shifts depending on current

environmental conditions. This includes the large body of work examining how exposure to toxins impacts immunity and disease e.g., (4,5). However, a large research gap exists when considering whether environmentally induced immune shifts are common to all individuals of a species or parceled into genotype-specific effects where only some individuals respond to cues (i.e., genotype-environment interaction; reviewed in Bolnick et al. (6).

The microbial environment surrounding an organism has strong impacts on host immunity. In mammals, the gut microbiome plays an important role in immune development and function, including maintenance of homeostasis and disease resistance (7). Much of what we know in this area comes from laboratory work on captive, inbred lines of house mice (*Mus musculus*). In addition to their limited genetic diversity, housing mice in sterile lab environments may limit our power to establish links between microbes and host immune phenotypes. Indeed, lab mice lack certain immune cell populations that are found in pet store mice (8) and have much lower levels of immune activation than wild animals due to limited pathogen exposure (9). Wild immune phenotypes can also be recapitulated through “rewilding” experiments that acclimate lab mice to natural outdoor enclosures. For example, wild-acclimated mice greatly increase granulocyte production and immune activation relative to laboratory animals (10,11). Similarly, inoculating lab mice with wild gut microbiomes lowered levels of inflammation and improved resistance to viral infection (12). Although most immunology research has focused on rodent models, teleost fish represent an emerging model for connecting environmental and microbial variation with immune development and function.

Fish models offer several useful avenues for studying immune variation. One clear benefit of these systems is that their mucosal organs are in constant contact with the aquatic environment and can quickly respond to environmental changes (13). Mucosal tissues such as respiratory surfaces harbor important microbial communities and act in the first line of immune defense (14). Numerous studies have examined how environmental factors such as salinity, pH, temperature, and pollutants affect fish mucosal immunity (reviewed in Morshed and Lee, (15)). Another large body of work addresses connections between pathogens and innate immunity, mostly focusing on organ specific immune responses (reviewed in Dezfuli et al, (16)). Apart from tissue or response specific studies, the ability to easily breed and rear fish across a spectrum of environments also offers research opportunities that are often not available with rodent models. For example, housing similar strains in lab, controlled outdoor (i.e., aquaculture), and wild settings, offers great opportunities for disentangling genetic and environmental effects on immunity. Moreover, in instances where genetics differences drive immune variation, several fish systems offer the opportunity to understand how and why these phenotypic differences evolved (17,18)

Threespine stickleback fish (*Gasterosteus aculeatus*) are well known as models for studying the process of adaptation via natural selection. This ancestrally marine species repeatedly colonized and adapted to freshwater ecosystems across the northern hemisphere as glaciers receded 11,000 years ago (19,20,21). Recent work suggests that population-specific immune divergence is an important factor in the adaptive radiation of stickleback into freshwater environments. Differences in innate immune function have been documented between numerous ecotypes, including marine-freshwater (22), sympatric benthic-limnetic (23), parapatric lake-stream ecotypes (24), and variation over larger, continental scales (25,26). While some of these studies include more modern measures of immune gene expression (18,23,27), many rely on two classic flow cytometric assays of immune cells collected from the head kidney (i.e., the primary hematopoietic organ). In one case, forward and side scatter are used to categorize populations and relative ratios of granulocytes and lymphocytes in the head kidney, with greater proportions of granulocytes suggesting greater investment in innate immunity (26). The second assay measures the oxidative burst response of granulocytes, where

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fluorescent dyes allow the quantification of reactive oxygen species (ROS) production after cells are exposed to an immune stimulant. (24,26). In general, populations with high natural macroparasite exposure tend to evolve higher proportions of granulocytes and larger oxidative burst responses (23,24). In addition to flow cytometric assays, the splenosomatic index (spleen mass/body mass, SSI) is frequently used to evaluate immune activity in fish, with an increased SSI corresponding to higher pathogen related immune response (28,29), potentially resulting from a greater need to filter circulating leukocytes. Another simple measure, the hepatosomatic index (liver mass/body mass, HSI) provides a proxy for individual condition because the liver is used to store fatty acids in fish (30).

We took advantage of threespine stickleback populations with recently evolved heritable differences in immune function to examine the effects of environment, most specifically microbial community, on host immunity. By rearing fish in a highly controlled laboratory setting, we were able to focus on two experimental variables: the effect of ancestry (i.e., host population) and the effect of water flow into tanks (i.e., constant versus intermittent flow). Based on previous results (18,26), we expected the cestode-resistant population to exhibit the highest immune response. We also hypothesized that variation in water flow rate would affect the composition of microbes in our fish tanks; specifically, we predicted that intermittent flow tanks would have more diverse microbial communities due to higher environmental stability. Finally, we predicted that housing adult fish in different water flow environments would reveal flexibility in adult innate immunity, likely because of different microbial communities. Specifically, if the intermittent flow tanks drive higher microbial diversity, fish housed in those tanks should also exhibit the largest immune responses. As most of our fish measurements focused on cellular immunity, to understand more generalized effects of water flow we also compared plasticity in two organs. We predicted that increased immune investment would correlate with increases in spleen size, and that decreased liver size would indicate increased energetic expenditure, potentially connected to immune activation.

Materials and Methods

Animal Care & Husbandry

Wild fish (F0-generation) were obtained from two sites on Vancouver Island, British Columbia: Roberts Lake (“resistant” population, 50.222, -125.541); Gosling Lake (“tolerant”, 50.061, -125.504); and from the Kenai River Estuary in southeast Alaska (“marine”, 60.542, -151.218). Offspring were generated via in-vitro fertilization (F1-generation) in the field in summer 2019 and fertilized eggs were shipped to the University of Wisconsin-Madison (ADFG license P-19-005; Canada: NA19-457335). F1 fish were bred in the lab after reaching maturity in 2021, and those offspring (F2) were used in this experiment. This breeding design should minimize maternal effects on measured phenotypes, as all parents shared the same laboratory environment. Fish were housed in 18L tanks in full-sibling groups (n/tank = 2 – 15) with a water temperature of 17°C. The room was kept on a 16:8 light cycle. Fish were fed daily with a mixture of freeze-dried bloodworms and mysis shrimp, and each tank had a section of PVC pipe and two artificial plants for environmental enrichment.

Acclimation Design

We used two water flow regimes to create different tank environments. Continuous-flow tanks had a volume of 18L and input flow rate of 1L/minute. The intermittent flow tanks had a volume of 23L, and water changes involved 1L/minute for 5 minutes every 12 hours. The entire volume of water was

replaced every 30 minutes in continuous flow tanks, as opposed to every 48 hours in intermittent flow tanks. The intermittent flow tanks were also provided standing bubbling filters to maintain water quality and promote microbial growth on the filter sponge. All tanks in the experiment shared a common water system, and water was run through a UV filter before recirculation to limit microbe movement between tanks. Due to a limited number of intermittent flow tanks, fry and young fish were all initially reared in constant flow tanks. All fish used for this experiment had been in their current tank for at least 11 months.

Fish ranged in age from 14 – 24 months at the start of the experiment and were randomly assigned to one of four treatments: continuous flow only ('c'), intermittent flow only ('i'), move from continuous to intermittent flow ('ci'), and move from intermittent to continuous flow ('ic'). After the latter two treatments were initiated, fish were maintained in the new tank for approximately eighteen weeks. After acclimation, we fasted fish for 24 hours and then euthanized via a combination of MS-222 overdose and pithing. Prior to dissection fish were weighed and standard length recorded. The liver and spleen were removed and weighed with an analytical balance (±0.0001), and the head kidneys (HKs) were removed for flow cytometric assays.

Flow Cytometry Assay

Maximum ROS production in HK cells was quantified via oxidative burst assays. Organs were placed in ice cold media (0.9× Roswell Park Memorial Institute medium containing 10% fetal bovine serum, 100 μM nonessential amino acids, 100 U/mL penicillin, 100 μg/mL streptomycin, and 55 μM β-mercaptoethanol) immediately after removal. Single cell suspensions were generated by manually disrupting and filtering the tissue on a cell strainer (35 μm) with a pipette. We washed the cell suspension once in 4 mL of cold media (300g, 4°C, 10 min), poured off most of the supernatant, and then resuspended the cell pellet in the remaining media. Live HK cells were counted using a hemocytometer (Hausser Scientific 3520) via Trypan blue exclusion (Corning 25-900-CI). Each individual had 2×10^5 HK cells distributed to three separate wells on a 96-well plate. Each well received either (1) 200 μL of HK medium or (2 & 3) HK medium with DHR-123 (ROS indicator fluorochrome, 2 μg/mL, Sigma D1054). Samples were incubated at 18°C, 3% CO_2 for 10 minutes. Next, PMA (Phorbol 12-myristate-13-acetat, 130 ng/mL; Sigma P8139) was added to well 3 to stimulate ROS production while wells 1 and 2 received equivalent volumes of HK media. Cells were incubated for a final 20 min at 18°C, 3% CO_2, placed in a sealed bag on ice for transport, and run on a ThermoFisher Attune NxT V6 flow cytometer.

Data Analysis

Flow cytometry data was analyzed in FlowJo (TreeStar). The magnitude of ROS production during oxidative burst assays was determined by comparing median fluorescence intensity of PMA-stimulated and unstimulated cells. The proportion of granulocytes and lymphocytes was determined by flow cytometry of the unstained, unstimulated HK cells and forward and side scatter gating described in Weber et al. (18).

All other data analysis was done in R v4.2. Because the amount of ROS produced by head kidney cells depended on time since exposure to PMA, we included flow cytometry sample order as a covariate. Specifically, we made separate linear models for PMA-stimulated and unstimulated cells with sample order as a random effect with R package *lme4* v35.5 (31) and used the model residuals as the corrected median fluorescence intensity. We then built a series of linear models for associations between four traits of interest (ROS production, percent granulocytes, liver and spleen

mass) and a suite of potential predictor variables: population, sex, family, tank environment (continuous/intermittent), flow treatment (c/ci/ic/i), movement between tanks (ic and ci groups were moved, c and i were not), body mass (only used for spleen and liver), and all relevant combinations of predictor variables and interaction terms. We used the R package *performance* v12.4 (32) to compare models and selected the model with the lowest AIC score for each response variable. The R package *emmeans* v1.11 (33) was used to estimate group averages and test for significant differences between multiple factors.

Tank 16S Sequencing & Analysis

We selected a subset of tanks for 16S sequencing to characterize microbial community differences between flow regimes. Five continuous and five intermittent flow tanks were sampled using a microbiome testing kit from Aquabiomics LLC (Junction City, OR). Two samples were taken from each tank. First, 60 mL of tank water was slowly pushed through a 22-micron mixed cellulose ester filter, followed by 2 mL of 90% ethanol; the filter was allowed to dry then sealed in a small plastic container. Second, each tank's water intake tube was swabbed vigorously with a cotton swab, the swab was dunked in a 1.5 mL cryotube filled with 90% ethanol, allowed to dry, then sealed in a plastic tube.

Sequence data were curated in R v4.2 with the *DADA2* pipeline (v1.16, 34). Reads were filtered and trimmed (command: 'filterAndTrim') with parameters maxN=0, truncQ=2, rm.phix=TRUE and maxEE=c(2,5). Paired reads were then merged (command: 'mergePairs') and chimera sequences were removed (command: 'removeBimeraDenovo'). R package *DECIPHER* (v.2.26, 35) was used to construct an amplicon sequence variants (ASVs) table (command: 'IdTaxa') after training on a reference dataset (SILVA 138 SSU; 36). Analyses of species richness and differences between communities were completed with the R package *phyloseq* (v1.42, 37).

Results

Tank Microbe Communities

Despite collecting and submitting 20 microbial samples for 16S library preparation and sequencing, we only recovered usable data from four tanks: three continuous flow and one intermittent. We were unable to acquire additional tank microbial samples because the experiment had concluded by the time that we learned of the sequencing error (i.e., we no longer had access to the relevant fish populations in each tank environment). Although the small number of tanks limits our confidence in measurements of microbial differences between water flow environments, we nonetheless identified several patterns. 2D ordination (Figure S1) separated the filter and swab samples along the first NMDS axis, and the intermittent flow tank separated from the three continuous flow tanks along the second axis. The intermittent flow tank also had the highest Shannon diversity index score (Figure S2), though it did not diverge greatly from the continuous flow tanks. We generated a list of the top ten ASVs that had the largest difference in read counts between the tank environments. ASVs in the genera *Sediminibacterium*, *Polynucleobacter*, and *Undibacterium* were overrepresented in both filter and swab samples in the intermittent tank (Table S1), whereas an ASV in the genus *Sphingopyxis* was underrepresented in the intermittent samples compared to continuous flow. Together, this suggests that the water flow regime likely altered the microbial communities where fish were housed.

Reactive Oxygen Species (ROS) Generation

The best fit regression model for ROS production included fixed effects for population and flow treatment, and family as a random effect (Table 2). The resistant stickleback population produced significantly higher ROS than tolerant (mean of 0.55 median fluorescence intensity vs -0.48 mfi, Cohen's d =-3.56, t-test p<0.00001, Table 1, Figure 2) or marine populations across all treatments (d =-1.72, p<0.00001). The tolerant population also produced less ROS than marine fish (d=-1.84, p<0.00001). These data support a strong role of population ancestry and evolutionary divergence in shaping oxidative burst capacity. Across all populations the c treatment had the lowest ROS production, which was significantly different from the i (d=-0.27, p = 0.004) and ci treatments (d=0.505, p = 0.0001) and marginally different from the ic treatment (d=0.37, p = 0.051). Fish in intermittent flow tanks tended to have greater ROS production than those in continuous flow (Figure S6, d=-0.11, p = 0.002). There was no effect of sex on ROS generation (p = 0.74).

Granulocyte:Lymphocyte Ratio

The best supported model for percent granulocytes included fixed effects for population, sex, and flow regime, and family as a random effect. Intermittent flow tanks averaged significantly higher proportions of granulocytes in the head kidney than continuous flow tanks (38.1% vs 34%; t-test p = 0.003, Cohen's d =-0.03, Table 1, Figure 3). The resistant population also had higher proportions of granulocytes than the tolerant (41.2% vs 34.4%, p = 0.02, d=-0.83) or marine (32.6%, p = 0.001, d=-1.05) populations. The latter two populations were not significantly different (p = 0.47).

Liver and Spleen Mass

Variation in spleen and liver mass was driven by fish mass, population, sex, and tank environment. Fish from the tolerant population had significantly smaller livers than resistant fish after controlling for mass (-0.44 vs 0.27 mass-scaled liver size, Cohen's d = -2.1, t-test p<0.00001, Table 1, Figure 4). The marine population was intermediate in liver size and not significantly different from either tolerant or resistant populations. Fish in the c treatment had significantly larger livers than the ci (0.16 vs -0.12, d=0.81, p=0.03) and ic treatments (0.16 vs -0.13, d=0.83, p=0.02) and were marginally larger than the ii treatment (0.16 vs -0.08, d=0.67, p=0.06). Males averaged smaller livers than females, but this difference was not significant in multiple comparisons (-0.04 vs 0.04, d = 0.23, p=0.16). The best-supported model for liver mass included population, sex, and flow treatment as fixed effects and family as a random effect.

The marine population averaged larger spleens than both resistant (0.16 vs -0.08 mass corrected spleen size, d = 0.49, p = 0.01, Figure 5) and tolerant populations (0.16 vs -0.08, d = 0.55, p = 0.006). Male fish had larger spleens than females (0.11 vs -0.11, d=0.45, p=0.01), and fish in intermittent flow had marginally larger spleens than continuous flow (0.06 vs -0.07, d = -0.28, p=0.05). The best-supported model for spleen mass included population, sex, and flow regime. Mass-corrected spleen size was not significantly correlated with either ROS production or granulocyte:lymphocyte ratio (Pearson's correlation coefficient, p>0.1). Mass-corrected liver size was positively correlated with ROS production (r=0.31, p=0.005), but not granulocyte:lymphocyte ratio (p=0.18).

Discussion

We leveraged evolved differences in immunity across stickleback populations to investigate how variation in the laboratory environment affects immune function. We found that simply transferring fish between tanks with constant or intermittent water flow, a relatively modest environmental change, led to significant changes in the inflammatory responses and composition of head kidney

cells. Intermittent water flow not only drove significantly higher levels of ROS production but also greater proportion of granulocytes–a category that includes neutrophils likely to drive strong oxidative burst responses. After eighteen weeks of exposure, the magnitude of both immune measures approximated levels observed in fish that had been housed in each environment for a long period. This demonstrates that environmentally induced flexibility in stickleback innate immune responses is a relatively rapid process.

Apart from classic measures of innate immunity, we also tested the impacts of water flow regime on liver and spleen size. Fish that ended the experiment housed in intermittent flow tanks had significantly higher SSI, consistent with a plastic shift to greater innate immune allocation. In contrast, there was no simple relationship between HSI and water flow treatment. Stable (non-transplant) rearing under constant water flow yielded significantly higher HSI than the other treatment groups. We had too few tanks available to perform sham transfers (i.e., c-c or i-i), so we cannot rule out a handling effect in the ci or ic treatments. However, this seems unlikely given that fish were allowed to acclimate to their new tanks for approximately eighteen weeks. The tolerant population had the lowest HSI overall. This was likely due to this population's fast growth rate, which is also associated with liver size in fish (38). Marine and resistant fish did not differ in relative liver size even though marine fish are significantly larger.

All populations showed a similar magnitude of flexibility for ROS production, granulocyte proportion, and SSI, and the heritable differences between populations were by far the strongest predictor of immune response (Table 2). Population accounted for 89% of the variance in ROS production, 42% of the variance in granulocyte proportion, and 43% of the variance in SSI. Several recent studies have highlighted the utility of population level comparisons of immune variation (e.g., 17, Robertson et al 2016, Weber et al 2022), but this remains a relatively neglected component of most experimental designs (Sasser et al 2023).

Variation in ROS production in these populations evolved recently in response to parasitism by the tapeworm *Schistocephalus solidus* which is not present in marine environments (18). We found a similar magnitude of difference between resistant and tolerant populations compared to previous work, although overall our measurements were lower than those reported in Weber et al (18). Our measures of granulocyte proportion were comparable to our previous results (18) but significantly higher than those reported in wild stickleback from Germany (24). This study was the first to include measures of ROS production in ancestral marine stickleback and demonstrated that the marine population is intermediate between the cestode resistant and tolerant freshwater populations.

Because our experiment was performed in a laboratory with highly controlled environments, we hypothesized that reducing the frequency of tank water changes would primarily lead to increased microbial diversity. Our results, with the caveat that they represent only a few tanks, support this prediction. The intermittent flow tank had the highest level of microbial diversity in swabs taken from the tank outflow pipes, and the second highest diversity from filtered water samples (Figure S2). Perhaps more striking was the divergence in microbe communities when accounting for ASV abundance. Whether considering microbes filtered directly from the water or from a swab of the tank outflow, 16S divergence along the first NMDS axis was strongly captured by flow regime (Fig S1). While the present study was a preliminary examination of whether variation in water flow could separately influence tank microbial environment and fish immunity, our positive results beg several questions: Do changes in tank microbial environment alter the fish microbiome and, if so, are the effects general or specific to areas such as the skin or gut? And are shifts in host immunity caused by microbial exposure, alterations in microbiome, or both events? More directed experiments are needed to address these questions, but our results do at least support a microbe-immune connection. The

higher ROS and granulocyte levels of head kidney cells from stickleback in intermittent flow tanks suggests an increased systemic immune stress (40). This is consistent with pathogen response, potentially mediating via migration of antigen presenting cells from the periphery to head kidney (41).

Ethics Statement

This experiment was reviewed and approved by the University of Wisconsin-Madison IACUC under protocol L006460-A01.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

1. Author Contributions

Conceived and designed the experiments: JW and CW. Performed the experiments, analyzed the data, and prepared the figures: CW. Wrote the first draft: CW and PC. Provided funding: JW. All authors contributed to the article and approved the submitted version.

2. Funding

This work was funded by a startup award from the University of Wisconsin-Madison Department of Integrative Biology (JNW) and the National Institute of General Medical Sciences (5R35GM142891; JNW)

3. Acknowledgments

We thank Dr J Benavente Paredes and M Chotlos for assistance with data collection; J Kosmopoulos and Dr K Anantharaman for advice on 16S data analysis; and E Kerns, K Yu, J Abels, and L Rippin for helpful feedback on this manuscript.

4. Data Availability Statement

The datasets generated for this study can be found on Dryad (https://doi.org/10.5061/dryad.0vt4b8hb8) and GenBank (link).

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Population	Treatment	ROS (mfi)	% granulocytes	Rel. liver mass	Rel. spleen mass
Marine	c	11.58±0.28 (11)	34.13±3.36 (15)	0.22±0.13 (22)	-0.06±0.12 (22)
	ci	11.88±0.17 (11)	31.42±6.08 (10)	0.06±0.12 (12)	0.34±0.37 (12)
	ic	11.96±0.22 (13)	29.93±3.97 (13)	-0.08±0.08 (17)	0.07±0.12 (17)
	i	11.68±0.20 (14)	35.02±2.76 (16)	0.01±0.07 (20)	0.19±0.17 (20)
Cestode tolerant	c	10.84±0.20 (21)	32.25±4.98 (17)	-0.27±0.11 (25)	-0.02±0.26 (25)
	ci	11.56±1.08 (4)	42.23±21.74 (3)	-0.42±0.24 (4)	-0.49±0.37 (4)
	ic	11.13±0.30 (8)	31.06±9.50 (5)	-0.28±0.11 (8)	-0.28±0.23 (8)
	i	11.57±2.02 (3)	41.38±14.88 (4)	-0.33±0.26 (6)	-0.16±0.19 (6)
Cestode resistant	c	12.51±0.41 (8)	37.09±8.25 (11)	0.34±0.47 (13)	-0.10±0.20 (13)
	ci	12.64±0.35 (8)	50.70±10.06 (8)	0.66±0.51 (7)	0.21±0.50 (7)
	ic	12.20±0.33 (3)	33.33±4.84 (3)	0.05±0.55 (3)	-0.05±0.26 (3)
	i	12.35±0.50 (9)	39.08±6.28 (10)	-0.07±0.07 (16)	-0.13±0.10 (16)

Table 1: trait means with 95% confidence interval by population and flow treatment (c = constant flow, i = intermittent flow, ci = moved from constant to intermittent, ic = moved from intermittent to constant). Sample size for each group in parenthesis. Relative masses are residuals from regression of organ mass on body mass.

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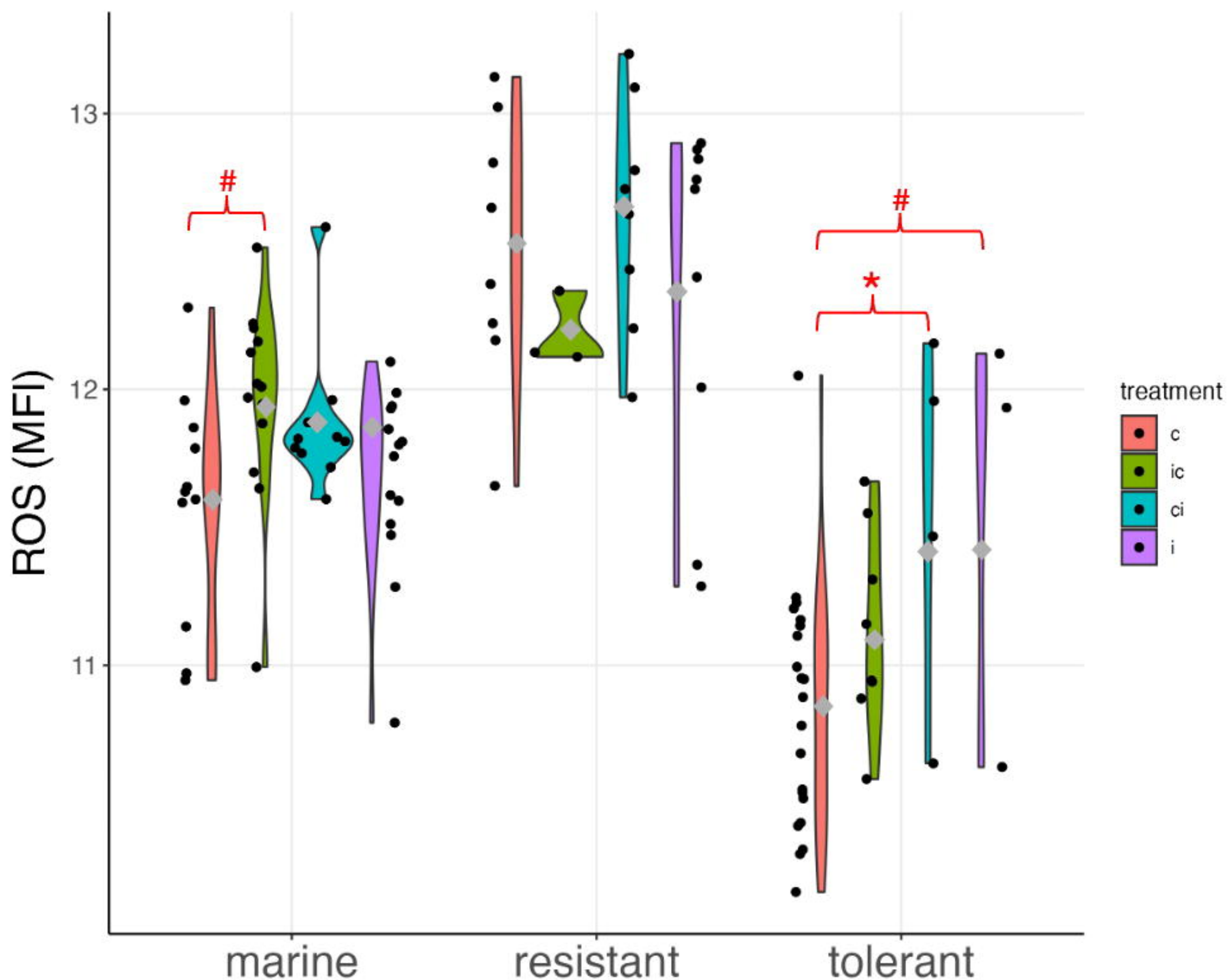
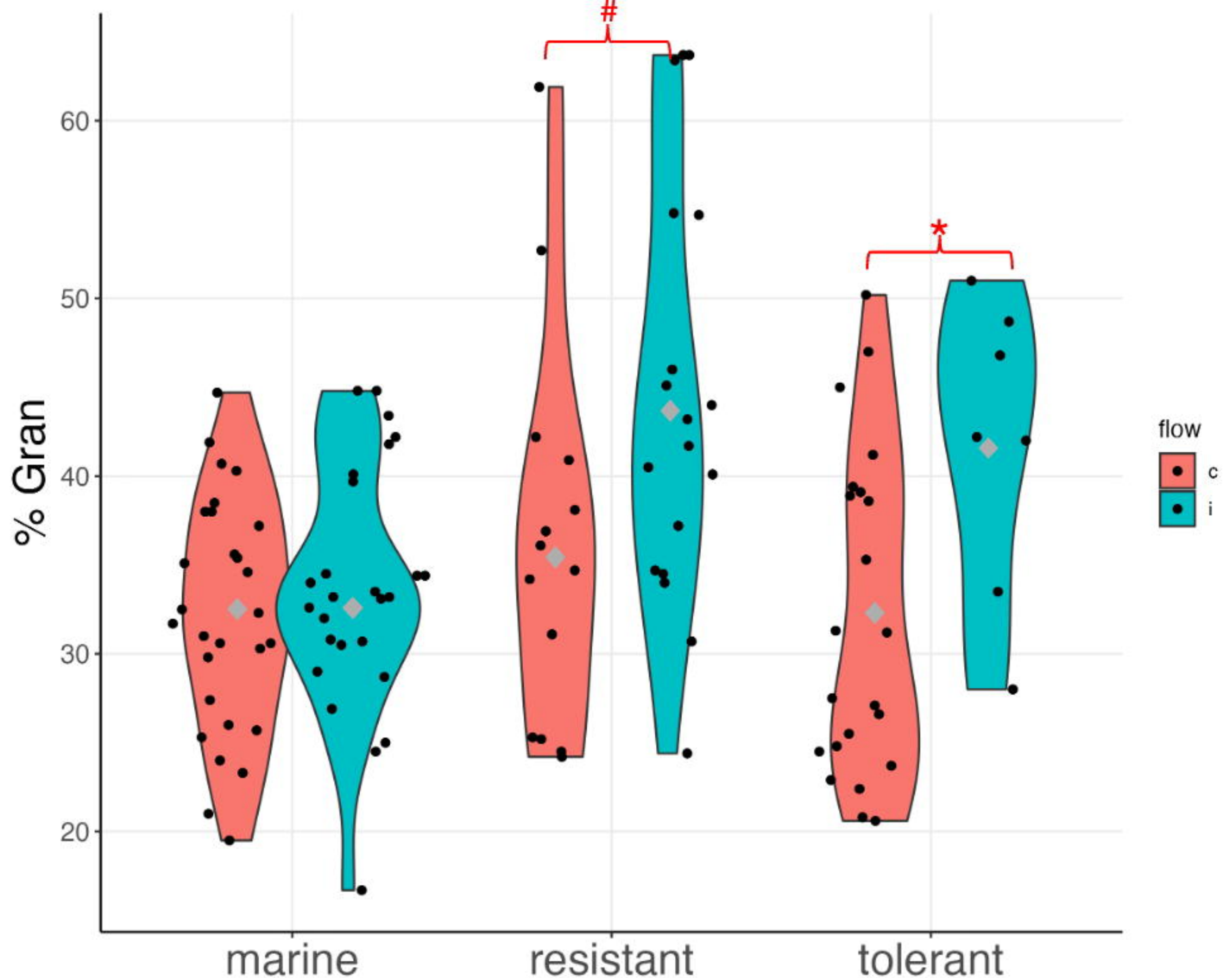
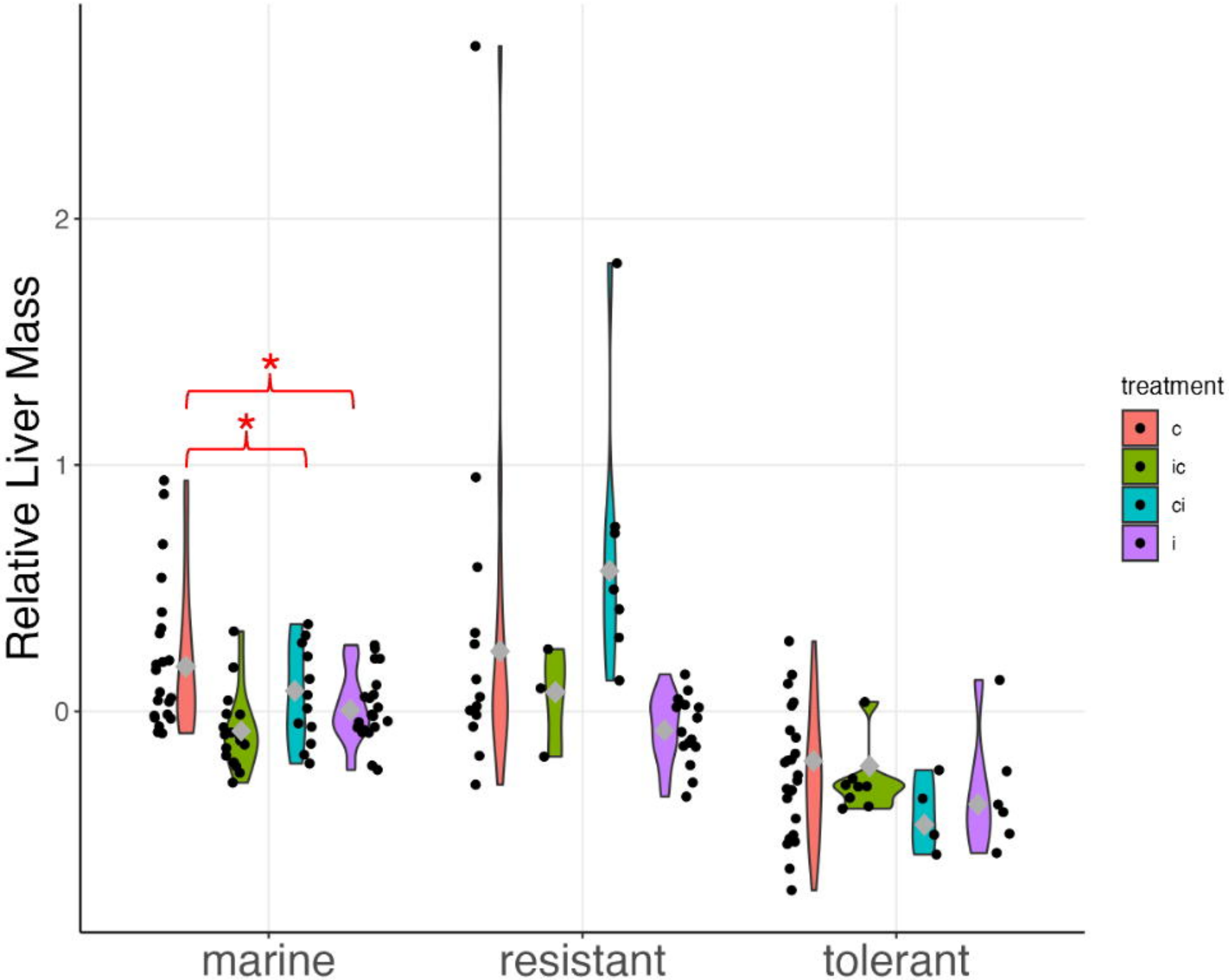
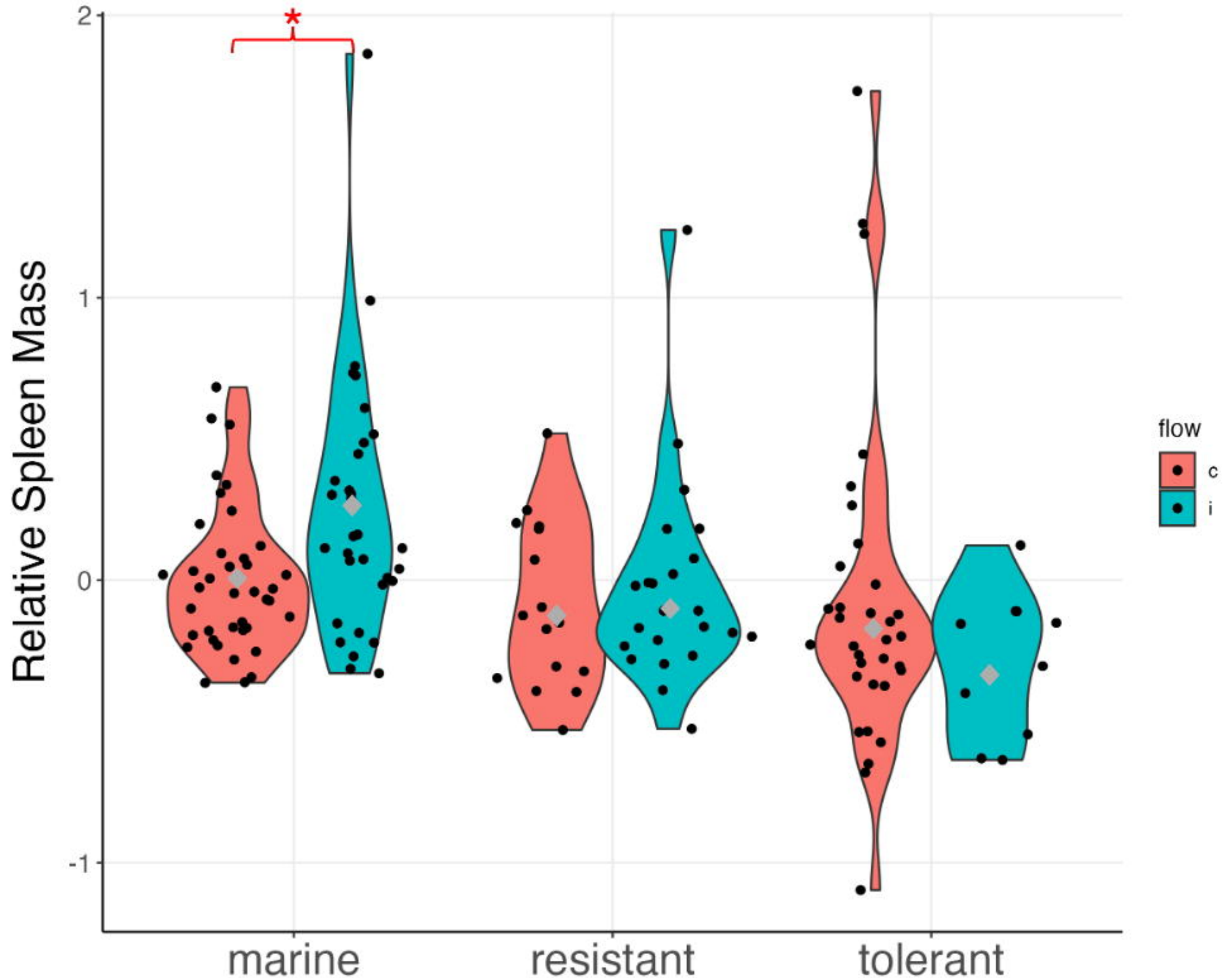
Trait	Predictor	Beta	SE	p	R^2
ROS	intercept	-0.06 (-0.38,0.24)	0.14	0.63	(0.61,0.70)
	pop-tolerant	-0.53 (-0.88,-0.18)	0.15	0.01	
	pop-resistant	0.50 (0.14,0.85)	0.15	0.01	
	treatment-ci	0.22 (0.05,0.39)	0.09	0.01	
	treatment-ic	0.18 (-0.01,0.38)	0.10	0.06	
	treatment-i	0.07 (-0.09,0.25)	0.09	0.38	
% Gran	intercept	-0.19 (-0.69,0.29)	0.19	0.35	(0.18,0.39)
	pop-tolerant	0.09 (-0.47,0.66)	0.23	0.70	
	pop-resistant	0.44 (-0.12,1.01)	0.22	0.10	
	sex-m	-0.1 (-0.27,0.05)	0.08	0.19	
	flow-int	0.21 (0.03,0.38)	0.09	0.02	
Liver mass	intercept	0.33 (-0.06,0.73)	0.16	0.09	(0.53,0.70)
	fish mass	0.95 (0.77,1.12)	0.09	0.00	
	pop-tolerant	-0.7 (-1.17,-0.23)	0.20	0.01	
	pop-resistant	0.1 (-0.36,0.56)	0.19	0.61	
	sex-m	-0.11 (-0.21,-0.02)	0.05	0.02	
	treatment-ci	-0.18 (-0.34,-0.02)	0.08	0.02	
	treatment-ic	-0.21 (-0.36,-0.06)	0.08	0.01	
	treatment-i	-0.15 (-0.29,-0.01)	0.07	0.03	
Spleen mass	intercept	0.07 (-0.06,0.20)	0.07	0.29	(0.40,0.40)
	fish mass	0.8 (0.62,0.98)	0.09	0.00	
	pop-tolerant	-0.43 (-0.64,-0.22)	0.11	0.00	
	pop-resistant	-0.24 (-0.4,-0.08)	0.08	0.00	
	sex-m	0.13 (0.00,0.26)	0.07	0.04	
	flow-int	0.13 (0.00,0.26)	0.07	0.05	

Table 2: coefficients from top-ranked model of each trait of interest. 95% confidence interval in parenthesis. 2


ROS (MFI)
13
12
11
#
#
*
marine
resistant
tolerant
treatment
c
ic
ci
i


#
*
% Gran
60
50
40
30
20
marine
resistant
tolerant
flow
c
i


Relative Liver Mass
2
1
0
*
*
marine
resistant
tolerant
treatment
c
ic
ci
i


Relative Spleen Mass
2
1
0
-1
*
marine
resistant
tolerant
flow
c
i