**Effects of genetics and early life mild hypoxia on individual growth and size variation
in gilthead sea bream (*Sparus aurata*)**

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Abstract

The present study evaluated, in an 18-month gilthead sea bream trial, the time course effects of genetics on individual size variation and growth compensation processes in the offspring of families selected by growth in the PROGENSA breeding program. Families categorized as fast, intermediate and slow growing had different growth trajectories with a more continuous growth in fast growth families. This feature was coincident with a reduced size variation at the beginning of the trial that clustered together the half-sib families sharing the same father. Correlation analysis evidenced that the magnitude of compensatory growth was proportional to the initial size variation with no rescaling of families at this stage. By contrast, the finishing growth depensation process can mask, at least partially, the previous family convergence. This reflects the different contribution across the production cycle of genetics and environmental factors in growth, production and welfare. How early life experiences affect growth compensatory at juvenile stages was also evaluated in a separate cohort, and intriguingly, a first mild-hypoxia pulse at 60-81 days posthatching (dph) increased survival rates by 10% preventing growth impairment when fish were exposed to a second hypoxia episode (112-127 dph). The early hypoxia experience did not have a negative impact on growth compensatory processes at juvenile stages. By contrast, a diminished capacity for growth compensation was found with repeated or late hypoxia experiences. All this reinforces the use of size variation as a main criterion for improving intensive fish farming or selective breeding into practice.

Keywords: Size heterogeneity; Growth compensation; Early life hypoxia; Selective breeding; Gilthead sea bream

Declarations

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Authors' contributions: JPS coordinated and designed the study; EP, ERM, FNC, PSM, JCG farmed fish from early life stages to the end of trial and participated in all samplings, JPS, ERM, EP analyzed the data, EP and JPS wrote the manuscript; all authors read, edited and approved the final manuscript.

Introduction

Growth trajectories of most animals are nearly identical when rescaled by body mass at maturity and time to reach mature size (Sibly et al. 2015). However, how metabolic processes fuel and regulate growth across life is under debate, though growth deceleration with increasing size appears mostly related to a limited diffusion rate of nutrients and oxygen (O₂) (Glazier 2010). Besides, not all individuals grow at the same rate and the large size variation of animals from the same cohort illustrates the great propensity of individuals to develop and grow at different rates (Pfister and Stevens 2002; Peacor et al. 2007; Teder et al. 2008). Growth variability is in fact a key ecological attribute differentiating individuals, populations and species (Dmitriew 2011; Vincenzi et al. 2014), contributing the habitat selection to maintain individuals on an optimal growth trajectory if habitat variation through life stages allows different ecotypes (Rosenfeld et al. 2015). Indeed, the different growth and life history traits of wild and farmed fish offer insights on the processes of growth regulation under natural selection, and the extent to which such processes can be altered by artificial selection or genetic modifications.

The overall high growth rates of domesticated fish stocks have been achieved by selection for high rates of energy intake and allocation of metabolic energy to rapid biomass production at the expense of a reduced energy expenditure on activity, predator avoidance or reproduction (Devlin et al. 2004; Sibly et al. 2015). Other factors such as rearing density, O₂ availability and social interactions also have major effects on growth phenotypic variability of farmed fish (Karplus et al. 2000; Barbosa et al. 2006; Azaza et al. 2013; Martos-Sitcha et al. 2019a). However, size heterogeneity is often ignored as a selection criterion in Mediterranean fish farming, though growth variability is considered one of the most important key performance indicators (KPI) to benchmark the efficiency of productive systems within the framework of the PerformFISH H2020 EU project (<http://performfish.eu>). Certainly, size heterogeneity is a good marker of environmental quality in amphibians and terrestrial livestock animals (Peacor and Pfister 2006; Peacor et al. 2007; Teder et al. 2008), and both in plant and animal genetics, the reduction of environmental variance produces animals that cope better with their environment (Mulder et al. 2008). Rabbits are a good example since the environmental variance of litter size is genetically determined and closely related to the viability of kits (Bodin et al. 2010; Blasco et al. 2017).

The growth rate of an individual organism has thereby ecological and evolutionary implications (Arendt 1997; Dmitriew 2011; Shelton et al. 2013), and many species of amphibians and fish establish dominance hierarchies, which usually reflect the larger size of socially dominant individuals (Symons 1971; Karplus et al. 2000; Grobler and Wood 2013). As a result of this, large size is often considered advantageous under interference, but disadvantageous under exploitative competition because of the

higher metabolic costs of most competitive individuals (Van Buskirk et al. 2017). To better understand the complex interplay and evolution of factors affecting fish size heterogeneity, we assessed in a common garden system the effect of genetics on the dynamics of size variation in fish families with different estimated breeding value for length at harvest (Perera et al. 2019). Additionally, given the pleiotropic effects of O₂ availability on growth and energy metabolism (Ratcliffe 2013), we also tested the impact of early life mild hypoxia on the achieved size variation. Both approaches provide new insights on the processes driving compensatory growth and size heterogeneity, serving as well to check the validity of size variation as a farming quality indicator in an important fish species for the Mediterranean aquaculture with a production of 185,980 annual tones in 2016 (FAO 2020).

Material and methods

Ethics

All procedures were carried out according to IATS-CSIC Review Board, European (2010/63/EU) animal directives and Spanish laws (Royal Decree RD53/2013) on the handling of experimental animals.

Long-term growth trial of selected families

Fish breeders belonging to the Spanish selection program of gilthead sea bream (PROGENSA) were used to produce families with different heritable growth as reported in Perera et al. (2019). Briefly, two mini-broodstocks, composed of nine (3 females, 6 males) and six (3 females, 3 males) fish with estimated breeding value for length at harvest of -0.9 ± 0.2 cm and $+0.9 \pm 0.7$ cm, were moved to spanning tanks to produce family crosses. Spawns occurred immediately after the formation of these two mini-broodstocks, and two spawns corresponding to three consecutive days were taken and incubated at 20 °C in cylinder conical tanks (400L). This procedure yielded 11 families that were grown up from early life stages to 199-228 g (September 2017-September 2018) at the experimental facilities of IATS-CSIC (Perera et al. 2019). In the present study, the trial was prolonged overwintering to better assess the time course effects of genetics on the achieved size and growth heterogeneity. Since no consistent differences in growth performance were observed with the two dietary formulations tested in the trial, all biometric data were analyzed as a single dietary treatment covering more than 2,500 individuals. All fish were individually tagged with passive integrated transponders (PIT) (ID-100A 1.25 Nano Transponder, Trovan), and genotyped individuals were reared together in replicate 3000-L tanks under natural photoperiod and temperature conditions (Fig. 1a) with well aerated seawater. The same family ratio was maintained in all replicated tanks, and the

concentration of water O₂ was always higher than 80 % saturation, varying fish density from 2.5 Kg/m³ at the beginning to 12 Kg/m³ at the end of trial. Fish were fed by automatic feeders 1-2 times per day and 3-7 days per week according to fish size and season, being the ration adjusted weekly to a level close to satiation. No significant mortalities occurred during all the trial and fish were weighed and measured individually each 2-4 months, using a FR-200 FishReader W (Trovan, Madrid, Spain) for data capture and pre-processing. For posterior analysis, three family groups with fast (c2c6, e5e2, e6e1, e6e2), intermediate (c2c5, c2c7, c4c7, e4e1, e4e2) and slow (c4c3, c4c5) growth rate phenotypes were established on the basis of their final weight in the 12-month trial of Perera et al. (2019).

Mild hypoxia experiences during early life stages

Gilthead sea bream larvae were transferred at 45 days post-hatching (dph) (May 2018) from the hatchery of Piscimar (Burriana, Castellón, Spain) to the facilities of IATS-CSIC. After an acclimation period of 15 days, 60 dph larvae (137 mg average weight) were distributed in six 90-L tanks (n = 150 larvae per tank) connected to two re-circulatory systems with control of water temperature (22-23 °C) and O₂ concentration (Martos-Sitcha et al. 2019a). Feeding was carried out to visual satiety several times per day with commercial pellets (0.2, 0.3, 0.5 mm Skretting Gemma Wean; 0.8 mm Skretting Perla Plus; 1.5 mm Biomar Intro Plus MT; 1.9, 3 mm Biomar EFICO YM 853). Water quality was daily checked and O₂ concentration was maintained high (85% saturation; 5.8 ppm) in normoxic tanks, whereas it was decreased gradually over the course of 24 h in fish exposed to mild hypoxia (50 %; 3.6-3.8 ppm). This reduced O₂ concentration was maintained for 21 days (60-81 dph) with the restoration of normoxia up to 112 dph. Then, fish from each tank were distributed in two additional tanks (40 fish per tank, 6.8-7.2 g average weight), and O₂ levels remained high or low for 15 days. This resulted in four groups of fish according to their O₂ availability history (NNN, normoxic; NNH, late mild hypoxic; HNN, early mild hypoxic; HNH: early and late mild hypoxic). At this end (July 2018), all fish were returned to normoxia, and 50 randomly selected fish (20-27 g) per experimental condition were individually tagged and reared together in a 3000-L tank until September 2018.

Statistical analysis

Statistical analyses were performed using SigmaPlot version 14.0 (Systat Software, San Jose, CA) with all P-values set to 0.05 unless otherwise stated. Normality and equal variance of data were tested by Shapiro-Wilk and Levene tests, respectively. Specific growth rates were calculated as $SGR = 100 \times (\ln \text{ final body weight} - \ln \text{ initial body weight}) / \text{days}$. Regression and Pearson correlation analyses were used to describe the relationships between SGR and individual size. The significance of regression slopes was tested by ANOVA and coefficient of determination were used as a measure of

relative goodness of fit of regression curves. Differences among slopes were assessed by ANCOVA, making all pairwise contrasts with a Bonferroni adjustment of the significance level to correct for multiple testing (Quinn and Keough 2002). Size heterogeneity was analyzed by measures of frequency distribution, coefficient of variation (CV) and skewness.

Results

The time course of growth compensation and depensation processes

The offspring of fish families selected for fast, intermediate and slow growth showed different growth trajectories. The highest difference in body weight among these groups increased from 15% in May 2018 to 26% one year later (April 2019), varying the average family weight at the end of trial from 258 g (slow growing families) to 347 g (fast growing families) (Fig. 1b). All families grew almost equally over the course of summer, but the fast growing ones continued to grow at a slow rate during overwintering whereas the slow growing families stopped growth completely or experienced a loss of weight, which was exacerbated during the second cold season (Fig. 1c).

During the first half of the trial (September 2017-July 2018), small fish of all families grew at a higher rate than their bigger counterparts, and consequently, a consistent negative correlation between SGR and initial body weight was found for two consecutive periods (Fig. 2a, b). The result was a reduction in size heterogeneity of all families in July 2018, as revealed by close-to-zero skewness of all frequency distributions of body mass (Fig. 3). This growth feature, referred as compensatory growth by Ricker (1958), was marginally affected by the genotype because the slopes of the group families categorized as slow, intermediate and fast growing phenotypes remained unaltered (Fig. 2a, b, Supplementary Table 1). However, this growth compensation appears to be body size dependent, because it stopped first in fast growing families with the achievement of 131-163 g of body weight before the summer growth spurt (Fig. 2c). By contrast, the reduction of size variability continued during the summer period in families with intermediate and slow growing features, until the final cessation of such growth compensation process with the achievement of 181-227 g of body weight during the second autumn season (Fig. 2d). Furthermore, the lack of growth compensation was followed overwintering by a growth depensation process in fast growing families (Fig. 2e). This resulted in a statistically significant positive correlation between SGR and initial body weight during the finishing period (November 2018-April 2019), which favored the growth of big fish in comparison to their small counterparts that shared an increased size heterogeneity. This feature was evidenced by differences in frequency distributions of body mass, with changes in skewness at the end of trial from positive values close to zero in slow growing families to negative values in intermediate/fast growth families (Fig. 3). Further analysis of compensatory growth during the first

half of the trial (September 2017 to July 2018) revealed that initial size variation (i.e. CV) in half-sib families sharing the same father was lowest in the fast growing families, but the close linear association between initial CV and compensatory growth revealed that it was almost equal in all families (Fig. 4).

Effects of early life mild hypoxia on growth, survival and size heterogeneity

In our experimental setup, episodes of mild hypoxia at 60-81 dph and 112-127 dph matched with key periods of early life stages, corresponding to nursery and pre-fattening, respectively (Fig. 5). The first mild hypoxia pulse did not affect growth rates, but enhanced by a 10% the survival rate of fish growing at very high rates ($\text{SGR} \geq 9$). With the recovery of normoxia, growth and survival rates were almost equal in NN and HN groups, but the response of these animals to a second episode of mild hypoxia reflected the history trajectory. Thus, growth rates were lowered by a 15% in NNH fish in comparison to NNN group, whereas both survival and growth in HNN and HNH did not differ significantly and remained high.

The early life episodes of mild hypoxia (May-July 2018) also modulated later the achieved compensatory growth during the second half of summer (August-September 2018) (Fig. 6). This process was dependent on the number of exposures and the time window in which they occurred. Thus, the trend for the slope of the regression line of SGR and initial body weight for the analyzed period was as follows $\text{NNN/N} < \text{HNN/N} < \text{NNH/N} < \text{HNH/N}$, with a clear reduction of the slope from -64×10^{-3} in NNN/N to -28×10^{-3} in HNH/N (Fig. 6, Supplementary Table 2). Accordingly, the final CV of body weight was lowest in NNN/N (7.83) and highest in HNH/N (13.6) groups. Likewise, the skewness changed from 0.006 in NNN/N fish to 0.626 in HNH/N fish, with intermediate values in HNN/N fish, as a result of notable changes in the frequency distribution of body weight (Fig. 7).

Discussion

The present study confirms and extends the idea that growth selection in the PROGENSA breeding program primes different growth trajectories and perhaps changes in standard metabolic rates as reported in salmon, where strong selection for attributes in one life stage strongly influences or constrains performance in another life stage (reviewed by Rosenfeld et al. 2015). Also, differences in energy partitioning on growth, maintenance, activity, and perhaps social behavior might lead the selection of animals that are not necessarily the fastest growing, but that have a positive effect on the group as a whole (de Goede et al. 2013; Devlin et al. 2004). Breeding based on behavioral properties are, however, difficult because the monitoring of social behavior is a costly and time consuming process, though the use of small smart accelerometers attached to the operculum now make feasible

to obtain reliable measures of both respiratory frequency and physical activity of livestock fish (Martos-Sitcha et al. 2019b; Rosell-Moll et al., unpublished results). Such approach contributes to build up individual phenotypic variations, affecting growth, production and welfare that are primarily conceptualized by changes in size variation. Certainly, fish welfare is compromised under intensive culture, and structural environmental enrichment has the potential to improve gilthead sea bream welfare (Arechavala-López et al. 2019). However, as pointed out by Balasch and Tort (2019), the heterogeneity of stress responses varies within individuals and stressors, and become multimodal in terms of typology, source and effects, being the intrinsic behavioral responses of each individual dependent on genetics and stressful experiences in early life as discussed below more in depth.

Individual growth variation is a fundamental component of evolution, but factors that similarly influence mean growth rate might affect differentially size variation (Peacor et al. 2007; Teder et al. 2008). This notion might be also extensive to livestock animals and early studies in gilthead sea bream indicated that size variation during early life is affected by both behavioral interactions and nutritional factors, though a general statement is that the improvement of culture conditions reduces the need for stressful and labor intensive grading operations (Goldan et al. 1997; Karplus et al. 2000). This is because different growth compensation mechanisms can become activated in a favorable environment. Herein, this growth feature was exemplified during the first-half of the trial by consistent negative correlations between growth rates and initial body weight. Besides, the achieved compensatory growth was mostly independent of genetics since the slopes of regression equations were almost equal in the families categorized as fast, medium or slow growth genotypes. Accordingly, at this stage, a close linear positive linear association was found for half-sib families between initial size variation (expressed as CV) and the achieved growth compensation (% of CV reduction). The same graphical representation displayed that the genetic component was a source of initial size variation, since the half-sib families are closely grouped each other rather than to other families. In any case, this growth compensation process is not extensive to all the production cycle as it stopped when a critical size was reached, matching well with the size (130-220 g) at which muscle hypertrophy is becoming the dominant mechanism of muscle growth in this species (Rowlerson et al. 1995). Therefore, until this end, the capacity to reduce size heterogeneity has the potential to be a suited KPI of the quality of farming conditions, helping us to discern improved or impaired management protocols at the production level regardless of genetic source. This was further supported by the observation that the capacity of growth compensation was compromised by early life environmental disturbances. Despite this, our results also highlighted that early life exposure to mild hypoxia serves to prepare the organism to better withstand stressful hypoxic episodes experienced later on.

A number of adaptive processes, displaying hypometabolic states with reduced risk of oxidative stress and improved exercise capacity, have been reported after acute or chronic hypoxia exposure in juveniles of gilthead sea bream (Magnoni et al. 2017; Martos-Sitcha et al. 2017; 2019a). Additionally, it is well known that early life hypoxia exposure increases the subsequent hypoxia tolerance at adult stage in zebrafish (Robertson et al. 2014). Likewise, acute hypoxic stimulus in non-hatched embryos displays a strong induction of glycolytic and glucose transport genes in trout at first-feeding (Liu et al. 2017). O₂ levels also shape metabolic traits in European sea bass (Cadiz et al. 2017, 2018), though in most cases the net balance is a growth depression (Zambonino-Infante et al. 2017) due to, among other factors, higher incidence of opercula abnormalities (Cadiz et al., 2018), delay in the maturation of digestive tract (Vanderplancke et al. 2015), with even transgenerational impairments of reproduction in the case of acute hypoxia episodes in medaka (Wang et al. 2016). However, in our experimental model, early exposure (60-81 dph) to mild hypoxia did not have a negative impact on individuals growing at high rates (SGR>9). Indeed, the reduction of O₂ availability increased the survival rates by about 10%. Since accelerated growth is by itself a source of oxidative stress (Costantini 2014), a possible explanation of this beneficial effect is that our hypoxia protocol helps to improve the oxidative balance. Certainly, the reduced O₂ availability would trigger a less active behavior with also less aggressive interactions. In any case, a second episode of mild hypoxia (112 to 127 dph) reduced growth rate only when fish never faced this environmental condition before, which is indicative of an effective environmental programming that produced different growth and survival phenotypes depending on the time window of the hypoxia episode. This also applied to compensatory growth feasibility, as the first early hypoxia episode did not compromise the growth compensation process during early juvenile stages. By contrast, growth compensatory constraints became evident with recurrent or late hypoxia episodes, which impaired, to a different extent, the capacity to reduce size variation during the dominant phase of muscle growth hyperplasia. However, it remains to be established the precise links between these phenotypic outputs and the early events, which affect both physiological (Pittman et al. 2013) and social behavior (Garduño-Paz et al. 2010; Bell et al. 2011; Langenhof et al. 2016) traits in fish.

Despite of the above limitations, it is plausible to postulate that the origin of the effects related to O₂ availability are of epigenetic nature. Indeed, it is known from studies in higher vertebrates that epigenetics plays a crucial role in the cellular response to hypoxia, involving the regulation of hypoxia-induced transcription factor (HIF) family, or the long maintenance of a hypoxia-adapted cellular phenotype after HIF has initiated the immediate response pathways (Watson et al. 2010). In fish, an early hypoxic environment affected the DNA methylation of specific loci in rainbow trout (Veron et al. 2018) and medaka (Wang et al. 2016). The link between coping style and reactivity of

the hypothalamic-pituitary-interrenal axis is also very important in terms of consequences for the maintenance of oxidative balance and down-stream effects related to changes in oxidative stress levels (Costantini et al. 2011). These issues warrant further examination, but intriguingly, measures of growth compensation processes are highly informative of disturbances experienced during early life, and would serve as a certificate of good management practices during the first half of the production cycle. By contrast, the genetic source of size variation operates earlier during development shaping the initial size variation of the population, and also during the finishing growth depensation process that apparently favors adult individuals with larger size (de March 1995; Sogard and Olla 2000). Such information is relevant to discriminate the main source of variation in growth rates and mean size along time or between different fish farms, helping to avoid the use of confounding criteria for improving fish management and selective breeding (Power et al. 2011). In any case, size variation is coming as a main criterion to evaluate and improve growth, production and welfare under the intensive Mediterranean fish farming.

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Figure captions

Fig. 1 Seasonal changes in temperature (solid line) and day length (dotted line) during the trial (a). Body weight of fish categorized as slow (SG), intermediate (IG) and fast (FG) growing families. Values are the mean of 3-5 replicate tanks (b). Changes in specific growth rates (SGR) of SG and IG fish, expressed as the percentage of FG fish in each period(c). Values are the mean $\pm$ sem of 3-5 replicate tanks

Fig. 2 Regression plots between initial body weight (IBW) and specific growth rates (SGR) in fish families of slow (SG), intermediate (IG) and fast (FG) growth rates at each period (a-e). Asterisks mean statistically significant correlations ($p < 0.05$). Within each graph, letters to the right of asterisks indicate statistically significant differences among slopes (ANCOVA)

Fig. 3 Frequency distribution of body weight (BW) at three sampling points of fish families growing at fast (a), intermediate (b) and slow (c) rates. Mean BW, skewness (Skw), and coefficient of variation (CV) are shown for each group and sampling point. Dotted lines mark the mean BW of the group with fast growth rates

Fig. 4 Regression plot between initial body weight (IBW) and specific growth rates (SGR) of slow, intermediate and fast growing families grouped into six half-sib families sharing the same father (a). Close positive linear regression between initial size variation and compensatory growth as a function of the reduction of coefficient of variation (b)

Fig. 5 Time windows of early life hypoxia exposure at different days posthatching (dph), leading to four experimental groups (NNN/N, NNH/N, HNN/N, HNH/N) (a). Overall body weight of all fish groups during the trial. Mean body weight at exposure times is indicated by dotted lines. Shaded area represents the period analyzed for compensatory growth (b). Data on survival and specific growth rates (SGR) after early life hypoxia exposure. Asterisks mean statistically significant differences ($p < 0.05$) (c)

Fig. 6 Regression plots between initial body weight (BW) and specific growth rates (SGR) at 147-191 days posthatching (August-September 2018) in fish exposed to none (NNN/N) (a), one early (HNN/N), one late (NNH/N) (b) or two (HNH/N) (c) mild hypoxia episodes during early life (60-81

dph and/or 112-127 dph). All correlations are statistically significant ($p < 0.05$). Letters to the right indicate statistically significant differences among slopes (ANCOVA) of all four groups

Fig. 7 Frequency distribution of body weight (BW) at 191 days post-hatching (dph) (September 2018) in fish exposed to none (NNN/N) (a), one early (HNN/N) or late (NNH/N) (b) or two (HNH/N) (c) mild hypoxia episodes during early life (60-81 dph and/or 112-127 dph). Mean BW, skewness (Skw), and coefficient of variation (CV) are shown for each group and sampling point

542 **Figure 4**

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Supplementary Table 1. Results of the regression analysis of specific growth rate (SGR) of gilthead seabream juveniles at the analyzed periods on the body weight at the beginning of each period. FG, IG, and SG mean fish families with fast-, intermediate-, and slow-growth rate, respectively. Letters next to the slopes mean statistically significant differences among slopes after ANCOVA. The Bonferroni adjustment of the significance level was used to correct for multiple testing. Only slopes from statistically significant correlations were compared.

Period	Group	N	F	P-value	Slope (x10 ⁻³)	R ²
Sept17-May18	FG	355	106.01	<0.001	-11.30 a	0.231
	IG	455	315.57	<0.001	-12.34 a	0.411
	SG	199	159.10	<0.001	-12.10 a	0.447
May18 -Jul18	FG	354	279.30	<0.001	-7.70 a	0.442
	IG	456	151.68	<0.001	-6.65 a	0.250
	SG	200	96.09	<0.001	-7.97 a	0.327
Jul18-Sept18	FG	347	2.66	0.104	-0.70	0.008
	IG	455	32.47	<0.001	-1.91 a	0.067
	SG	199	35.56	<0.001	-2.98 a	0.153
Sept18-Nov18	FG	331	0.77	0.381	0.17	0.002
	IG	259	0.05	0.830	-0.05	0.000
	SG	190	0.66	0.419	-0.20	0.003
Nov18-Abr19	FG	341	12.88	<0.001	0.19	0.037
	IG	261	1.27	0.261	0.07	0.005
	SG	190	0.36	0.551	0.04	0.002

Supplementary Table 2. Results of the regression analysis of specific growth rate (SGR) of gilthead seabream juveniles, from 147 days post-hatch (dph) (August 2018) to 191 dph (September 2018), on body weight at the beginning of that period. Fish experienced none (NNN/N), one early (HNN/N), one late (NNH/N), or two (HNH/N) mild hypoxia conditions during early farming (60-81 dph and/or 112-127 dph). Letters next to the slopes mean statistically significant differences among slopes after ANCOVA. The Bonferroni adjustment of the significance level was used to correct for multiple testing.

Period	Group	N	F	P-value	Slope (x10 ⁻³)	R ²
Aug18- Sept18	NNN	31	59.30	< 0.001	-63.90 a	0.672
	HNN	30	23.68	< 0.001	-54.60 ab	0.458
	NNH	48	35.51	< 0.001	-40.30 ab	0.436
	HNH	48	19.26	< 0.001	-28.10 b	0.295