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Association among growth, food consumption-related traits and amylase gene polymorphism in the Pacific oyster Crassostrea gigas

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Abstract:

To examine further a previously reported association between amylase gene polymorphism and growth in the Pacific oyster Crassostrea gigas, ecophysiological parameters and biochemical and molecular expression levels of α-amylase were studied in Pacific oysters of different amylase genotypes. Genotypes that previously displayed significantly different growth were found to be significantly different for ingestion and absorption efficiency. These estimated parameters, used in a dynamic energy budget model, showed that observed ingestion rates (unlike absorption efficiencies) allowed an accurate prediction of growth potential in these genotypes. The observed association between growth and amylase gene polymorphism is therefore more likely to be related to ingestion than to absorption efficiency. Additionally, relative mRNA levels of the two amylase cDNAs were also strongly associated with amylase gene polymorphism, possibly reflecting variation in an undefined regulatory region, although no corresponding variation was observed in specific amylase activity. Amylase gene sequences were determined for each genotype, showing the existence of only synonymous or functionally equivalent non-synonymous polymorphisms. The observed associations among growth, food consumption-related traits and amylase gene polymorphism are therefore more likely to be related to variation in the level of amylase gene expression than to functional enzymatic variants.

Keywords: α-amylase • bivalve • gene expression • ingestion

1. Main text

Gene polymorphism has important implications for variation of biological traits and is associated with economically important characters in many farmed species. In Pacific oyster *Crassostrea gigas*, an association was recently established between growth and a PCR-RFLP polymorphism at the two closely linked genes (*AMYA*, *AMYB*) encoding α-amylase, a key enzyme for starch digestion (Prudence *et al.* 2006). In other animals, such a relationship between *amylase* gene polymorphism and growth is thought to be due to variation in digestive parameters (food conversion in the chicken, Hughes *et al.* 1994) or in biochemical characteristics of this enzyme; as found for amylase quantity in *Drosophila* (Hickey 1981) and the chicken (Hughes *et al.* 1994).
To further explore the association between *amylase* gene polymorphism and growth in *C. gigas*, the present study recorded ecophysiological parameters and *amylase* expression at the mRNA and enzyme levels, in four PCR-RFLP *amylase* genotypes of a full-sib family that had shown different growth performances under field conditions (family 2 in Prudence *et al.* 2006). *A1/2,B2/2* was the fastest-growing genotype (total and flesh weight), *A1/5,B1/6* had the lowest weights, and *A1/1,B1/2* and *A2/5,B2/6* showed intermediate values after one year of standard rearing in one site.
For each genotype (defined using *Eco*RI, as in Sellos *et al*. 2003) 150 animals were conditioned in duplicate raceways of 5µm-filtered seawater (106L/h) at 15±0.5°C and fed a 20% *Chaetoceros gracilis* – 80% *Rhodomonas salina* (containing 3.4% DW of starch) diet at a daily ration equal to 10% dry weight algae/dry weight oyster. Ecophysiological parameters (Savina & Pouvreau 2004) were periodically measured in each raceway and in a control raceway without oysters. Each tank was connected to a system that took continuous measurements of dissolved oxygen and chlorophyll (as in Le Moullac *et al*., 2007). This provided 10 days of continuous data per animal which were then corrected to 1g dry tissue weight for the following parameters: clearance rate, from fluorescence recordings ($L.h^{-}1.g^{-1}$), used to obtain ingestion rate (cells $h^{-1}.g^{-1}$); oxygen consumption ($mgO_2.h^{-1}.g^{-1}$); and absorption efficiency (%) of organic matter from food ingested. All rates were converted into energy values using the conversion coefficients: 0.32 µJ/cell for *C. gracilis*, 1.58 µJ/cell for *R. salina* and 13.8 J/mg O_2 to estimate scope for growth ($J.h^{-1}.g^{-1}$). After 2 weeks, 30 oysters per genotype were randomly sampled (15 per raceway) and weighed (total and wet flesh weights). Their digestive glands were dissected, immediately weighed, pooled (6 pools of 5 glands meaning 3 pools per raceway), frozen in liquid nitrogen, crushed to a fine powder at -196 °C with a Dangoumau mill and stored in liquid nitrogen until RNA extraction and enzymatic assay. Total RNA was isolated using Extract-all reagent (Eurobio) at a concentration of 1 ml/50 mg powder and treated with DNAseI (Sigma, 1U/µg total RNA). RNA concentrations were measured at 260 nm (1 OD= 40 µg/ml RNA), and quality verified with a denaturing agarose gel. Two µg of total RNA were reverse-transcribed and amplified in triplicate by real time PCR according to Huvet *et al*. (2004) with specific primers (*AMYA* forward: 5'caacggggacatgagcatt3', reverse: 5'cgttacggaaggcaacca3'; *AMYB* forward: 5'cgcgtcacggacttcatt3', reverse: 5'cagcgtcattggagttaggc3'). The calculation of relative mRNA levels was normalized to *Elongation Factor I* (primers in Fabioux *et al*. 2004) as no significant differences of Ct values were observed for *EFI* between genotypes (*P*= 0.134, CV= 2.7%).
Amylase activity and apparent Michaelis-Menten constant (K_M) were evaluated on the 6 replicate pools according to Prudence *et al*. (2006). In addition, to further document polymorphism besides *Eco*RI, and take into account its extremely high magnitude at the nucleotide level (Sauvage *et al*., 2007), segments of cDNA *A* and *B* were amplified in both parents and a few offspring using PCR with specific primers (*AMYA* F 5'agcacgggagacggcaat3', R 5'gcaaccactgggtcatcact 3'; *AMYB* F 5'cgaccctgggaggaaagata3', R 5'tgaggggtcccatccagc3'). These segments, of 1037 and 905 bp for *A* and *B* respectively, contained the PCR-RFLP segment and were larger than the alleles reported in Sellos *et al*., (2003). PCR products were subcloned with a TOPO-TA cloning kit (Invitrogen) and both strands sequenced using a capillary sequencer system ABI 3730 XL, sequencing kit ABI BigDye Terminator and universal primers. Sequences were paired-assembled (Genbank accession numbers EU477250 - EU477255), translated into deduced proteins and aligned using ClustalW.
All statistical analyses, including tests of normality, one-way ANOVAs and multiple comparison tests (Tukey's HSD method) were performed using STATGRAPHICS software. As no significant difference was observed between replicate raceways for weights, mRNA level or amylase activity, these data were pooled.

Total and flesh weights, recorded on the same full-sib family one year later than those reported by Prudence *et al*. (2006), agreed with previous results. Genotype *A1/2,B2/2* was heaviest, followed by

genotypes *A2/5,B2/6* and *A1/1,B1/2* (which were significantly different in our study unlike in Prudence *et al*. 2006), and lastly *A1/5,B1/6* (Table 1).

Significant ecophysiological differences were observed between the *amylase* genotypes (Table 1): the two fastest growing genotypes (*A1/2,B2/2*; *A2/5,B2/6*) showed the highest values of ingestion, absorption efficiency (energy input) and oxygen consumption (energy consumed); the two slowest growing genotypes (*A1/1,B1/2*; *A1/5,B1/6*) showed the opposite. These variations in ingestion and absorption efficiency led to differences in estimated scope for growth (energy available for growth or stored as reserves) with the highest values for *A1/2,B2/2* and *A2/5,B2/6* genotypes despite their higher oxygen consumption. However, although the two fastest growing genotypes showed the highest values of absorption efficiency, our results suggest that absorption efficiency is not the main cause of growth variation between genotypes. Indeed, *A2/5,B2/6* had a 10% higher absorption efficiency than *A1/2,B2/2,* whereas weights (total and wet) of *A2/5,B2/6* were lower than those of *A1/2,B2/2* (Table 1). Finally, when the extreme values of ingestion obtained for the *A1/2,B2/2* and *A1/5,B1/6* genotypes (4.72 and 4.32 cells $h^{-1}.g^{-1}$, respectively) were put into the *C. gigas* dynamic energy budget model (Pouvreau *et al*. 2006), the predicted weights fitted precisely with the growth differences reported by Prudence *et al.* (2006) (Figure 1). Measured variations in ingestion are therefore sufficient to accurately predict potential growth of these *amylase* genotypes.

AMYA mRNA levels, showed two significantly different groups. Transcript levels for the *A1/2* and *A2/5* haplotypes were twice those of the other haplotypes (Table 1). For *AMYB* mRNA level, the linked haplotypes *B2/2* and *B2/6* in the same two groups were also significantly different, but had the lowest transcript levels: mean value around 400 times lower than for *AMYA*, leading to the total *amylase* mRNA levels being similar to those of *AMYA* (Table 1). The minor contribution of *AMYB* mRNA level to total mRNA level calls into question the role of this gene or suggests tissue specific transcription, as the *AMYB* gene was observed not only to be significantly expressed in digestive gland (Huvet *et al,* 2003) but also in labial palps (Sellos *et al*. 2003). These high variations observed in mRNA level suggest that relative mRNA levels were also strongly associated with the gene polymorphism, possibly reflecting variation in an undefined regulatory region. However, they did not lead to significant differences in specific amylase activity between the genotypes (Table 1). Only total amylase activity (taking into account specific amylase activity and size of the digestive gland) displayed a trend related to growth variation, also clustering with *amylase* mRNA level, ingestion and absorption efficiency (P=0.049, although no statistical groups formed following Tukey's HSD test at the 5% level).

Amylase sequences were determined for each genotype (see supplementary material). A total of 23 and 14 mutations were characterized in sequences *A* and *B*, respectively: 81% appearing to be synonymous mutations. The 7 non synonymous mutations observed, 3 for *AMYA* and 4 for *AMYB*, are neutral modifications located outside the α-helical and β-sheet enzyme structure. The genotypes should therefore possess amylases of similar physiochemical nature, which is supported by the identity of the apparent Michaelis-Menten constants between genotypes (Table 1). As a result, the observed association between growth, food consumption-related traits and *amylase* gene polymorphism is more likely to be related to variation in the level of *amylase* gene expression than functional enzymatic variants. The absence of correlation between gene expression and specific activity (as already observed by Huvet *et al*. 2003) suggests we should study modes of amylase regulation further.

Although the present results are not fully conclusive, the suggestion of associations linking growth, consumption related traits and *amylase* expression with an *amylase* polymorphism are important for the improvement of oyster production. If variation in weight gain is partly due to an increase in absorption efficiency, this would be a very useful criterion to select for improved oyster growth in a breeding program. However, we observed that weight gain mainly results from an increase in ingestion, so one might ask whether selective breeding (see Ward *et al*. 2005) could improve oyster growth in sites where food availability is a limiting factor. This is notably the case in France where increased aquaculture production of *C. gigas* has been shown to be negatively correlated with productivity due to competition for food in some grow-out sites (Héral *et al.* 1986). Our data highlight the importance of environmental conditions such as food availability when selecting to improve growth-related traits in oyster. Genotype x environment interactions can indeed be generated by highly plastic genetic correlations in this species (Ernande *et al*. 2004; Evans & Langdon, 2006). A more comprehensive Quantitative Trait Loci analysis of growth in this or other families is needed to reveal other genes that could contribute to determining growth rate in oyster.

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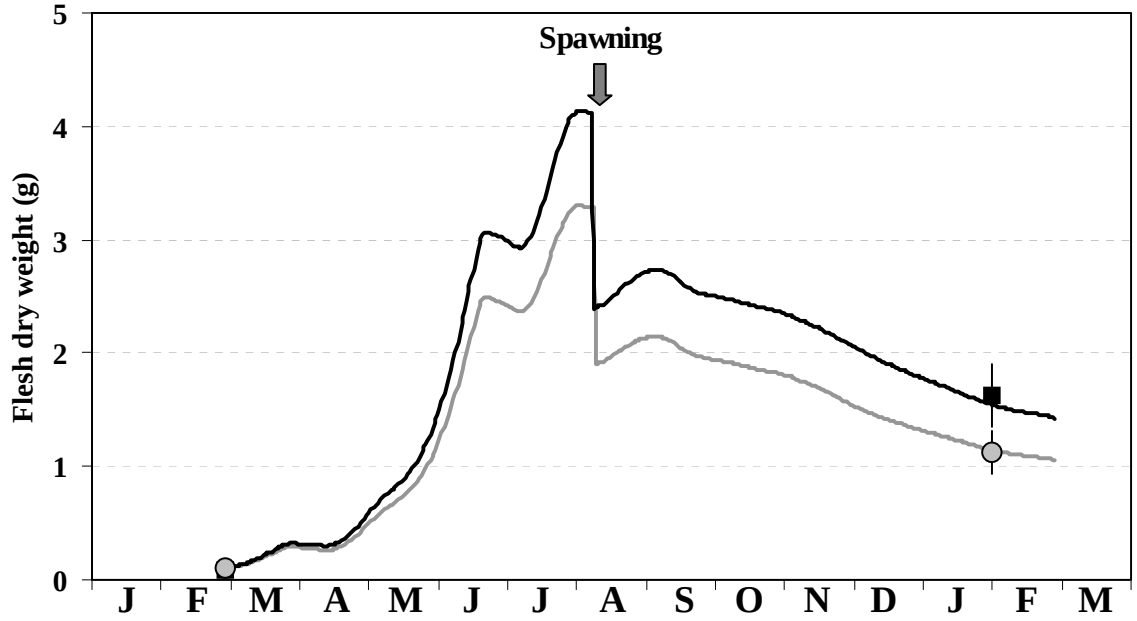
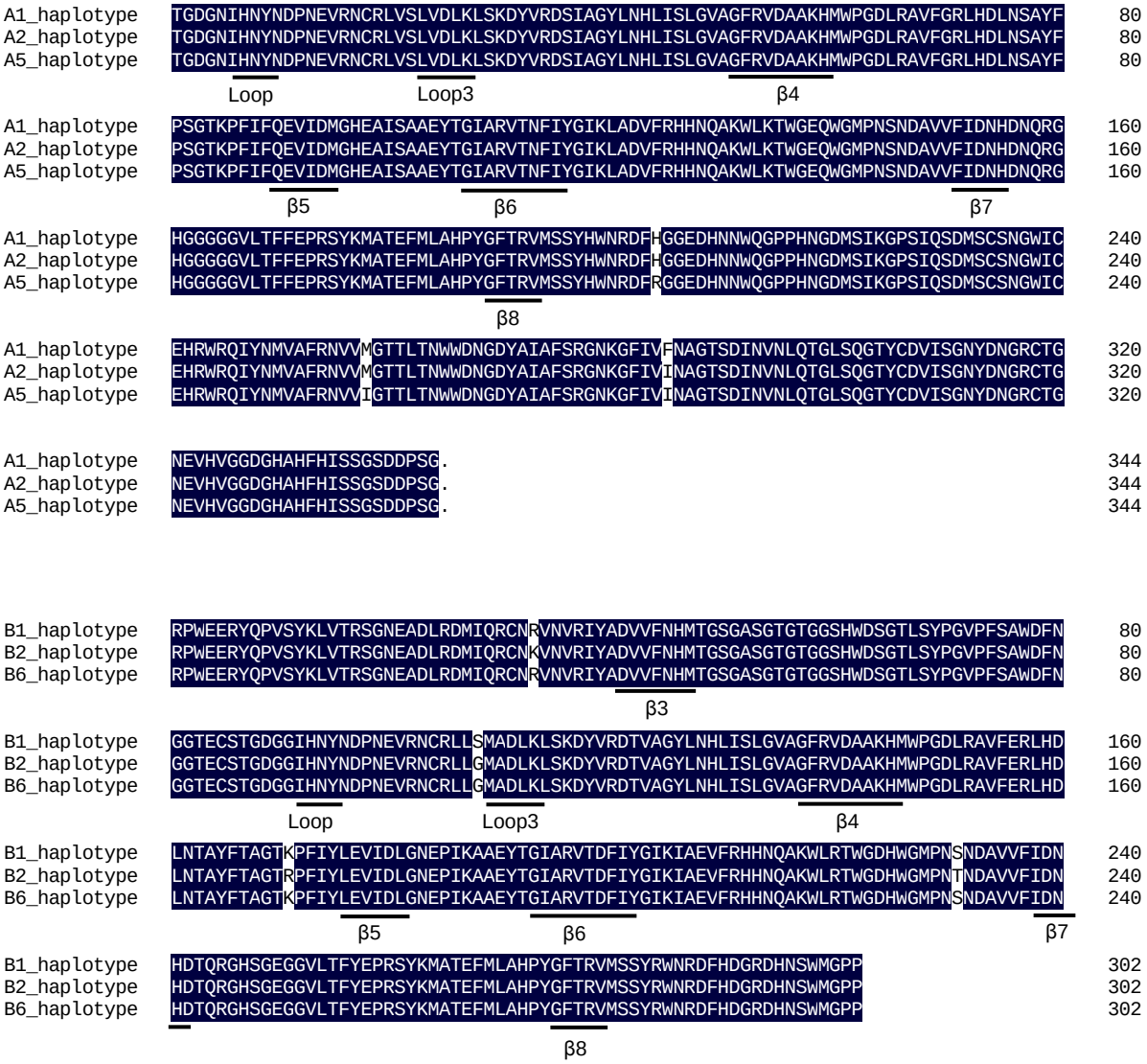
Figures

Figure 1. Simulation of growth (flesh dry weight) for the *amylase* genotypes *AMYA1/2,AMYB2/2* (in black) and *AMYA1/5,AMYB1/6* (in grey) using their estimated differential ingestion rate (ingestion = 4.72 and 4.32 cells $h^{-1}.g^{-1}$ respectively; Table 1) in a deterministic model (dynamic energy budget model proposed for *Crassostrea gigas* by Pouvreau et al. (2006)). To validate the model, observed weights (available for one year from a standard rearing conducted in the field by Prudence et al. (2006)) are given (black and grey points) at the beginning and end of the simulation.



Supplementary material. Alignement of the amylase *AMYA* and *AMYB* enzymes deduced from nucleotidic sequences of three haplotypes per cDNA (A1, A2, A5 and B1, B2, B6) sequenced in four *amylase* genotypes (*AMYA1/1,AMYB1/2; AMYA1/2,AMYB2/2; AMYA1/5,AMYB1/6; AMYA2/5,AMYB2/6*). Amino acids identical between haplotypes are indicated in black and those with a similar physiochemical nature in white. Underlined sequences correspond to the common secondary structure of animal amylases.



Tables

Table 1. Weights, ecophysiological parameters, amylase properties and *amylase* gene expression of four *amylase* genotypes (genes *A* and *B*) within one oyster family.

[1]Ingestion is expressed in millions of cells ingested $h^{-1}.g^{-1}$ of wet flesh, Oxygen Consumption in mgO_2 $.h^{-1}.g^{-1}$ of wet flesh and Absorption Efficiency in %. These parameters were converted to $Joule.day^{-1}.gram^{-1}$ of wet flesh to estimate the Scope For Growth ($J.h^{-1}.g^{-1}$).
[2]Specific amylase activity is given in UI/mg soluble protein and apparent Michaelis-Menten constant (K_M) in mg/ml of starch. Total amylase activity = specific amylase activity x digestive gland weight.
[3]the amount of *amylase* transcripts of *A* or *B* relative to *elongation factor I* transcript.
± values are standard deviations. Multiple comparisons were made between genotypes using Tukey's HSD method at the 5% level and homogenous groups share letters. The Scope for Growth is estimated and was therefore not tested statistically.

	Weights (gram)			Ecophysiological parameters[1]				Amylase parameters[2]			*amylase* mRNA level[3]	
	Total weight	Wet flesh	Digestive gland	Ingestion	Oxygen consumption	Absorption efficiency	Estimated Scope for growth	Specific activity	Total activity	K_M	Total mRNA level	composed of above: mRNA A below: mRNA B
AMYA1/1, AMYB1/2	43.64 ± 15.25 C	7.52 ± 2.96 C	0.94 ± 0.27 B	4.32 ± 0.01 B	0.079 ± 0.006 C	65.71 ± 9.54 B	48.8	0.58 ± 0.06	0.55 ± 0.10	0.32 ± 0.07	0.074 ± 0.023 B	0.073 ± 0.022 0.00036 ± 0.00006
AMYA1/2 AMYB2/2	64.93 ± 16.16 A	11.54 ± 3.17 A	1.18 ± 0.36 A	4.72 ± 0.05 A	0.084 ± 0.010 B	66.37 ± 8.64 B	54.7	0.64 ± 0.06	0.76 ± 0.13	0.35 ± 0.06	0.131 ± 0.022 A	0.131 ± 0.021 0.00019 ± 0.00007
AMYA1/5, AMYB1/6	40.52 ± 16.21 C	7.10 ± 2.72 C	0.91 ± 0.31 B	4.32 ± 0.01 B	0.083 ± 0.009 B	66.24 ± 4.65 B	47.3	0.65 ± 0.03	0.60 ± 0.22	0.31 ± 0.05	0.073 ± 0.024 B	0.073 ± 0.023 0.00029 ± 0.00010
AMYA2/5, AMYB2/6	57.91 ± 12.55 AB	10.56 ± 2.87 AB	1.07 ± 0.33 AB	4.73 ± 0.05 A	0.094 ± 0.012 A	72.67 ± 4.35 A	57.3	0.65 ± 0.05	0.70 ± 0.28	0.30 ± 0.11	0.137 ± 0.026 A	0.137 ± 0.026 0.00018 ± 0.00003