Potential of biochemical indicators in egg quality assessment in spotted wolffish (*Anarhichas minor*)

Presented to
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Introduction

Fish farming industry is the food production sector having the strongest growth in the world. This young industry is still searching for means to improve production, its success resting on its ability to produce high-quality products. Survival rate is obviously the most limiting factor for mass production. In marine fish species, the larval stage is the major bottleneck that producers have to undergo. It has been shown that larval performances are dependent on egg quality (Kjorsvik et al. 2003, Lamarre et al. 2004). Study of egg quality received considerable attention (Kjorsvik 1994, Pavlov & Moksness 1994a, Shields et al. 1997, Halfyard et al. 2000, Neidig et al. 2000, Wendling et al. 2000, Tveiten et al. 2001, Lahnsteiner & Patzner 2002), because survival rates until first feeding shows an uncontrolled variability within and among batches.

Obtaining a better knowledge of intrinsic physiological parameters of the developing egg and the identification of physiological components responsible for egg viability, are steps that could lead to better utilisation of hatcheries enabling the early detection and removal of poor quality egg batches. Recent studies have focused on the link between biochemical parameters and quality of the egg. Yolk composition and a few enzyme activities have already been correlated with the egg viability in lake trout *Salmo trutta lacustris* (Lahnsteiner et al. 1999), in gilthead sea bream *Sparus aurata* (Carnevali et al. 2001), in sea bass *Lates calcarifer* (Nocillado et al. 2000) and some cyprinids species (Lahnsteiner et al. 2001). First developmental stages in fish are characterised by high growth rates, an energetically demanding process (Blier & Pelletier 1997), implying enzymatic systems for metabolic processes and digestive functions, as well as all the machinery for protein synthesis and deposition. In the wolffish (*Anarhichas lupus*), it has been reported that larval performances are related to metabolic and digestive functions prior to first feeding (Lamarre et al. 2004). Considering that the development of metabolic pathways begin during embryonic development, egg quality could be linked to these metabolic characteristics.

Spotted wolffish (*Anarhichas minor*) is a promising species for cold-water aquaculture (Le François et al. 2002) and is at commercial stages in Norway and pre-pilot in Iceland and Canada (province of Québec). Principal characteristics that makes spotted wolffish a good candidate are; 1) larvae are well-developed at hatching (Falk-Petersen & Hansen 2001); 2) larvae readily feed on exogenous food at hatching eliminating the need of live feed; and 3) wolffish show remarkable juvenile growth performances in captivity. However, eggs survival is subject to high variability, limiting the

establishment of a reliable supply of juveniles for the industry (Falk-Petersen et al. 1999, Lamarre et al. 2004). Spotted wolffish is an interesting model for physiological studies given their relatively large egg size which allows the measurement of several parameters in a single individual egg.

The aim of this study is to identify biochemical indicators of egg quality from the energy metabolism and digestive capacities pathways of the spotted wolffish during embryogenesis. The following metabolical pathways will be investigated : 1) capacities to use carbohydrates (pyruvate kinase), amino acids (aspartate amino transferase) and fatty acids (hydroxy acyl coA dehydrogenase) for energy production, 2) aerobic (citrate synthase) and anaerobic (lactate dehydrogenase) modes of energy production, and 3) digestive capacities of proteins (trypsin like proteases).

Material and methods

Sample collection

Spotted wolffish eggs were obtained from a captive broodstock reared at the Troms Steinbit AS fish farm (Senja, Norway). Eggs were obtained from 12 females (6 - 7 years old), artificially fertilised and incubated at mean temperatures of 6.6±0.6 °C (mean±sd). During embryonic development, egg samples from each spawn (n = 5 eggs, a spawn is referred to as a batch of eggs coming from a single female) were taken from the incubation trays at day 110 after fertilization, just before hatching. They were kept at –80 °C until laboratory analysis. Survival was measured at hatching (% of the fertilized eggs that survived until hatching). These survival measurements were then considered as measure of egg quality and used as response variable when trying to relate egg quality to enzyme activity.

Enzyme activity measurements

Enzymatic analyses were performed at the Centre Aquacole Marin de Grande-Rivière (Québec, Canada). Activities were determined on individual eggs. Whole eggs were weighed (EW, mg) and homogenized in 9 volumes of Tris-HCl buffer 100 mM (pH 7.5) using a 2 ml glass potter. Crude homogenates were centrifuged in a Sigma 3K30 refrigerated centrifuge (13 000G, 30 seconds); the supernatant was collected for analysis. A portion of the homogenate (total dilution 1/200) was refrozen at –20°C for protein determinations, 300µl was conserved at –80°C for RNA/DNA content as well as relative mRNA concentration of structural and functional proteins (in progress).

For all enzymes, a Lambda 40 UV/VIS dual beam spectrophotometer (Perkin Helmer) equipped with a

water-jacketed cell holder connected to a VWR Scientific 1186 circulating refrigerating water bath set at 15 °C was used. All assays were performed in duplicate and activity results were expressed in U g egg^{-1} and in U g protein^{-1}, were one U (units) represents one µmol substrate transformed by minute (µmol min^{-1}) . The assay conditions were as follows :

Pyruvate kinase, PK (EC 2.7.1.40) : 50 mM imidazole-HCl, 10 mM MgCl2, 100 mM KCl, 5 mM ADP, 0.15 mM NADH, 5 mM phosphoénolpyruvate, 0.6 U ml-1 lactate dehydrogenase, pH 7.4 (Pelletier et al. 1994). Extinction coefficient of NADH at 340 nm : 6.22 mmol^{-1} cm^{-1}.

Aspartate amino transferase, AAT (EC 2.6.1.1) : 50 mM potassium-phosphate, 0.025 mM pyridoxal phosphate, 0.32 mM NADH, 10 mM α-ketoglutarate, 22 mM aspartate, 0.6 U ml^{-1} malate deshydrogenase, pH 7.4 (Pelletier et al. 1994). Extinction coefficient of NADH at 340 nm : 6.22 mmol^{-1} cm^{-1}.

Trypsine like proteases, TRY (EC 3.4.21.4): 0.2 M Tris-HCl buffer pH 8.0, 50 mM Calcium chloride, 10 µM Na-Benzoyl-L-arginine 4-nitroanilide hydrochloride.

β-Hydroxy acyl CoA dehydrogenase, *HOAD* (EC 1.1.1.35) : 100 mM triethanolamine-HCl, 5 mM EDTA, 1 mM KCN, 0.115 mM NADH, 0.05 mM acetoacetyl CoA, pH 7.0 (Thibeault et al. 1997). Extinction coefficient of NADH at 340 nm : 6.22 mmol^{-1} cm^{-1}.

Lactate dehydrogenase, LDH (EC 1.1.1.27) : 100mM potassium-phosphate, 0.16 mM NADH, 0.4 mM pyruvate, pH 7.0 (Thibeault et al. 1997). Extinction coefficient of NADH at 340 nm : 6.22 mmol^{-1} cm^{-1}.

Protein assays

Total protein content of whole eggs were determined using the bicinchoninic acid method (Smith et al. 1985).

Results

This section will be divided in three parts; the first part will be devoted to data description; in part II, activity of each enzymes will be used as a single explanatory variable of survival (egg quality). Simple regression analysis will be used for this task since both variable are on ratio scale and the expected error distribution is normal. The third part of the result section will be devoted to the analysis of the effects

of two or more enzymes as explanatory variable of egg quality. In this section, multiple regression analysis will be used.

PART I

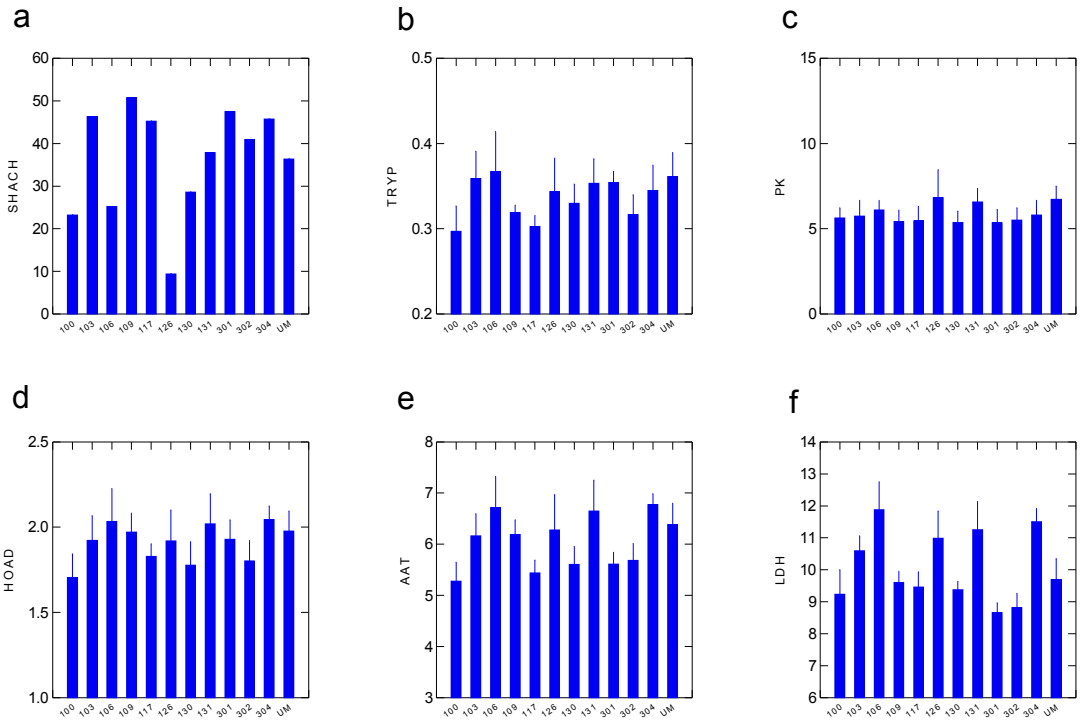

Figure 1 Distribution of the variable measured in relation to familiy number (X axis); a) survival rate, b) Trypsine activity in relation to family, c) Pyruvate kinase (PK), d) β-Hydroxy acyl CoA dehydrogenase (HOAD), e) aspartate aminotransferase (AAT) f) lactate dehydrogenase (LDH). Enzyme activities are presented in internationnal unit and survival is %.

The survival rate presents a marked difference between families and range from 9.4 to 50.7% (figure 1a). Mean enzyme activities present variation between families as shown in figure 1 and general descriptives statistics are presented in table 1.

Table 1 Descriptive statistics of enzymatic activities and survival rates

	TRYP	PK	HOAD	AAT	LDH	SHACH
N of cases	60	60	60	60	60	60
Minimum	0.21	3.53	1.23	3.98	7.00	9.40
Maximum	0.44	12.17	2.28	7.76	13.91	50.75
Mean	0.34	5.88	1.91	6.06	10.09	36.43
Standard Dev	0.06	1.61	0.26	0.91	1.51	12.02
Coefficient of variation	0.16	0.27	0.14	0.15	0.15	0.33

PART II

The goal of this section is to verify whether egg quality depends on the activity of a single enzyme. The

performed analysis will be simple regression using survival at hatching (SHATCH) as response variable (ratio scale with % as units) and enzyme activity as explanatory variable (ratio scale with international enzyme units U as unit). The model will be SHATCH = $\beta_0 + \beta_{enz}$ x enz + $\in$, where *enz* will be replaced by the activity of each enzyme (total of five regressions). The H_a/H_o testing is appropriate in this situation since the tests are used in order to see if the variation in the survival rate can be related to the enzymatic activity or if it is just due to chance; H_a being $\beta_{enz} \neq 0$ and $H_0 = \beta_{enz} = 0$. For all the regressions, the theoretical distribution of t will be used with $\alpha = 0.05$. For all regressions, a residual analysis was performed and graphs are presented (except the residuals vs residuals at lag 1, the residuals were independants in all performed analysis).

Trypsin

output 1

```
MTB > Regress 'SHACH' 1 'TRYP'
The regression equation is
SHACH = 37.2 - 2.3 TRYP


Predictor         Coef      SE Coef           T         P
Constant        37.204        9.804        3.79     0.000
TRYP            -2.31        28.68       -0.08     0.936
S = 12.12       R-Sq = 0.0%       R-Sq(adj) = 0.0%


Analysis of Variance
Source             DF           SS           MS          F         P
Regression          1          1.0          1.0       0.01     0.936
Residual Error     58       8521.9        146.9
Total              59       8522.8
```

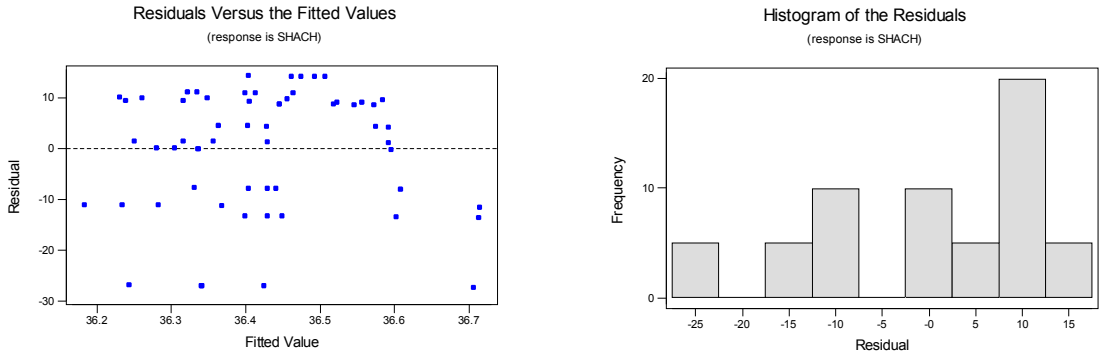

Figure 2 Residuals in relation to fitted value (left) and frequency distribution of the residuals (right) of the regression analysis of survival on trypsin activity

The residuals of this regression are homogeneous (figure 2, left) but are not considered as normal since their distribution is not centered on zero and strongly skewed on the right (figure 2, right). The high P-Value and the elevated number of data are sufficient although for accepting the null hypothesis; there is no effect of the activity of trypsin on the survival at hatching, despite the violation of the normality assumption.

Pyruvate kinase (PK)

output 2

```
MTB > Regress 'SHACH' 1 'PK';
The regression equation is
SHACH = 43.9 - 1.27 PK

Predictor        Coef     SE Coef           T        P
Constant       43.886       5.865        7.48    0.000
PK            -1.2696      0.9629       -1.32    0.193

S = 11.94       R-Sq = 2.9%      R-Sq(adj) = 1.2%

Analysis of Variance

Source             DF          SS          MS          F        P
Regression          1       248.0       248.0       1.74    0.193
Residual Error     58      8274.8       142.7
Total              59      8522.8
```

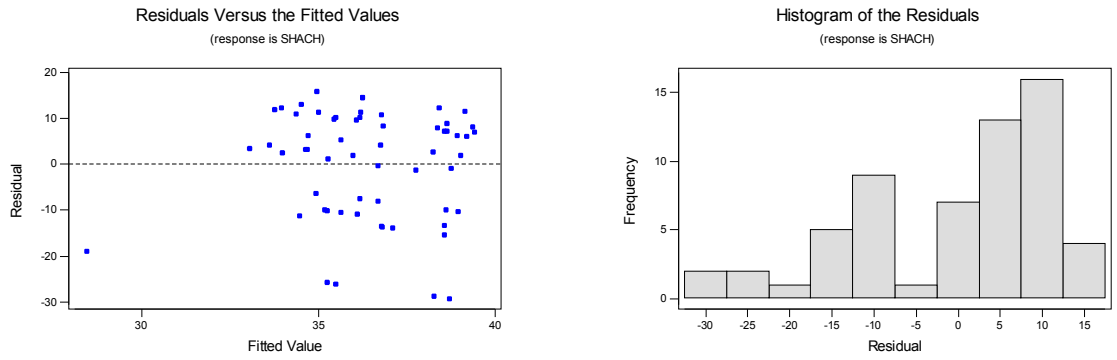

Figure 3 Residuals in relation to fitted value (left) and frequency distribution of the residuals (right) of the regression analysis of survival on pyruvate kinase activity

The residuals of this regression are homogeneous (figure 3, left) but are not considered as normal since their distribution is not centered on zero and strongly skewed to the right (figure 3, right). The elevated number of data is sufficient although for accepting the null hypothesis; there is no effect of the activity of PK on the survival at hatching, despite the violation of the normality assumption.

β-Hydroxy-acyle-coA-dehydrogenase (HOAD)

output 3

```
MTB > Regress 'SHACH' 1 'HOAD'
The regression equation is
SHACH = 28.2 + 4.29 HOAD

Predictor        Coef     SE Coef           T        P
Constant        28.24       11.49        2.46    0.017
HOAD            4.286       5.957        0.72    0.475

S = 12.07       R-Sq = 0.9%      R-Sq(adj) = 0.0%

Analysis of Variance

Source             DF          SS          MS          F        P
Regression          1        75.4        75.4       0.52    0.475
Residual Error     58      8447.4       145.6
Total              59      8522.8
```

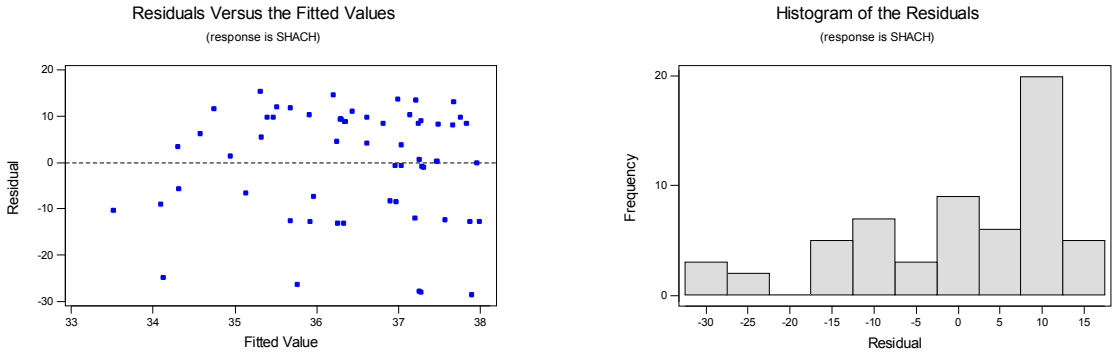

Figure 4 Residuals in relation to fitted value (left) and frequency distribution of the residuals (right) of the regression analysis of survival on β-Hydroxy-acyle-coA-dehydrogenase activity

Once again, the residuals are homogeneous and are not centrally distributed around zero. The P-value is high enough to state that there is no effect of HOAD on egg quality.

Aspartate amino transferase

output 4

```
MTB > Regress 'SHACH' 1 'AAT';
The regression equation is
SHACH = 37.1 - 0.12 GOT

Predictor        Coef     SE Coef           T        P
Constant        37.14       10.67        3.48    0.001
AAT            -0.118       1.741       -0.07    0.946

S = 12.12       R-Sq = 0.0%      R-Sq(adj) = 0.0%

Analysis of Variance

Source             DF          SS          MS          F        P
Regression          1         0.7         0.7       0.00    0.946
Residual Error     58      8522.2       146.9
Total              59      8522.8
```

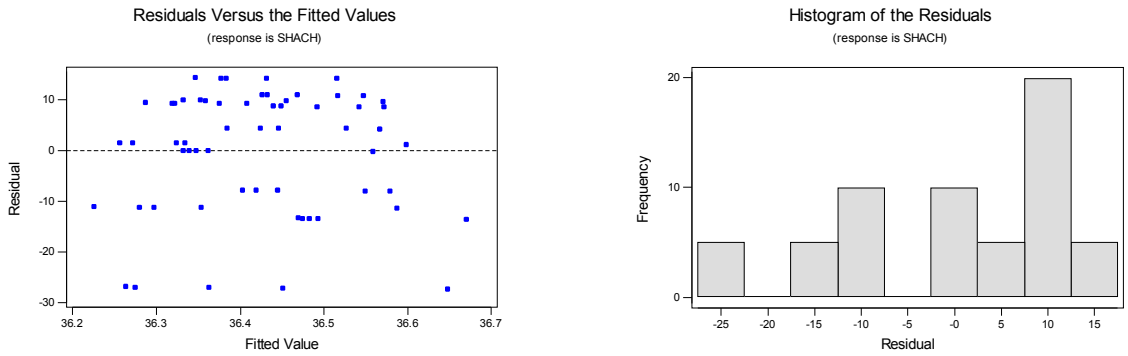

Figure 5 Residuals in relation to fitted value (left) and frequency distribution of the residuals (right) of the regression analysis of survival on aspartate amino transferase activity

The residuals are homogeneous but not normal. The P-value is close to 1 and the number of data high enough so it is safe to conclude that there is no effect of AAT on egg quality despite the violation of assumptions.

output 5

```
MTB > Regress 'SHACH' 1 'LDH';
The regression equation is
SHACH = 52.1 - 1.56 LDH

Predictor        Coef     SE Coef           T        P
Constant        52.12       10.48        4.97    0.000
LDH            -1.555       1.027       -1.51    0.135

S = 11.89       R-Sq = 3.8%      R-Sq(adj) = 2.1%

Analysis of Variance

Source             DF          SS          MS          F        P
Regression          1       324.0       324.0       2.29    0.135
Residual Error     58      8198.9       141.4
Total              59      8522.8
```

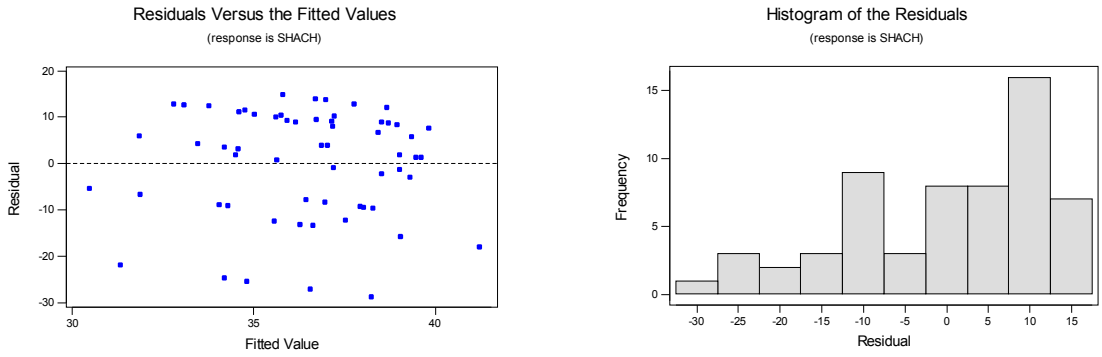

Figure 6 Residuals in relation to fitted value (left) and frequency distribution of the residuals (right) of the regression analysis of survival on lactate dehydrogenase activity

the residuals are homogeneous but not centrally distributed around 0. The P-Value is somewhat close

to α but again the number of data is high enough to accept the null hypothesis stating that there is no effect of LDH on egg survival despite the violation of assumptions.

PART III

In the second part, an attempt will be made to explain the survival at hatching by a model based on more than one enzyme activity. The test used in this case is a multiple regression using SHATCH as response variable and the enzymes as explanatory variables. The question is whether the survival rate at hatching is related to the composit effect of several enzymatic activities. In order to find the most relevant predictors to include in the multiple regression, the stepwise regression routine of Minitab was used as an exploratory tool. The basic procedures involve 1) identifying an initial model (step 0), 2) repeatedly altering the model at the previous step by adding or removing a predictor variable in accordance with the stepping criteria and method, 3) stepping end when there is no longer possible alterations given the stepping criteria. The stepping criteria is based on the P-value of the predictor. The forward and backward entry methods are simple model-building procedures. For the forward entry procedure, at each Step after Step 0, the entry statistic is computed for each effect eligible for entry in the model. If no effect has a value on the entry statistic which exceeds the specified critical value for model entry, then stepping is terminated, otherwise the effect with the largest value on the entry statistic is entered into the model. For the backward removal methods, the initial model includes all effects specified to be included in the design for the analysis. The initial model for these methods is therefore the whole model. At each Step after Step 0, the removal statistic is computed for each effect eligible to be removed from the model. If no effect has a value on the removal statistic which is less than the critical value for removal from the model, then stepping is terminated, otherwise the effect with the smallest value on the removal statistic is removed from the model. In order to find the most relevant predictors to include in the multiple regression, the stepwise regression routine of Minitab was used with forward selection and an α to enter of 0.25. Then the results were confirmed using the same routine with backward elimination and an α to remove of 0.1. This technique was used since there is a general mistrust in stepwise procedures, especially when using predictors presenting collinearity (that we have here!). When collinearity is present between two predictors, only one will be in the final model. It can be problematic but in our situation, if two predictors are bringing the same information, only one is sufficient (cost/benefit is important). Using forward followed by backward procedure with tighter criteria should lead to the identification of the best possible model.

Forward Stepwise Regression: SHACH versus TRYP; PK; HOAD; AAT; LDH

```
MTB > Stepwise 'SHACH' 'TRYP' 'PK' 'HOAD' 'AAT' 'LDH';
SUBC>   Forward;
SUBC>   AEnter 0.25;
SUBC>   Constant.

Forward selection.  Alpha-to-Enter: 0.25
Response is  SHACH   on  5 predictors, with N =   60

    Step          1        2        3
Constant      52.12    39.00    31.51

LDH            -1.6     -3.4     -4.2
T-Value       -1.51    -2.64    -3.49
P-Value       0.135    0.011    0.001

HOAD                    16.6     37.6
T-Value                 2.26     4.19
P-Value                0.028    0.000

PK                              -4.3
T-Value                        -3.52
P-Value                        0.001

S              11.9     11.5     10.5
R-Sq           3.80    11.71    27.69
R-Sq(adj)      2.14     8.61    23.81
C-p            16.8     12.9      2.8
```

Backward Stepwise Regression: SHACH versus TRYP; PK; HOAD; AAT; LDH

```
MTB > Stepwise 'SHACH' 'TRYP' 'PK' 'HOAD' 'AAT' 'LDH';
SUBC>   Backward;
SUBC>   ARemove 0.1;
SUBC>   Constant.

Backward elimination.  Alpha-to-Remove: 0.1
Response is  SHACH   on  5 predictors, with N =   60

    Step          1        2        3
Constant      32.13    32.23    31.51

TRYP            -37      -38
T-Value       -0.85    -0.88
P-Value       0.400    0.384

PK             -4.1     -4.1     -4.3
T-Value       -3.33    -3.38    -3.52
P-Value       0.002    0.001    0.001

HOAD           43.7     43.3     37.6
T-Value        2.54     3.90     4.19
P-Value       0.014    0.000    0.000

AAT            -0.2
T-Value       -0.03
P-Value       0.978

LDH            -4.1     -4.1     -4.2
T-Value       -2.50    -3.46    -3.49
P-Value       0.015    0.001    0.001

S              10.6     10.5     10.5
R-Sq          28.68    28.68    27.69
R-Sq(adj)     22.08    23.50    23.81
C-p             6.0      4.0      2.8
```

In both cases, the results were the same and three enzymes were retained for the multiple regression analysis, LDH, PK and HOAD. The model tested will be as follow;

$SHATCH = \beta_0 + \beta_{LDH} \times LDH + \beta_{PK} \times PK + \beta_{HOAD} \times HOAD + \in$

output 7

```
MTB > Regress 'SHACH' 'LDH' 'PK' 'HOAD'

The regression equation is
SHACH = 31.5 - 4.16 LDH - 4.25 PK + 37.6 HOAD

Predictor        Coef     SE Coef          T        P
Constant        31.51       10.87       2.90    0.005
PK             -4.251       1.208      -3.52    0.001
HOAD           37.627       8.981       4.19    0.000
LDH            -4.164       1.192      -3.49    0.001
S = 10.49       R-Sq = 27.7%     R-Sq(adj) = 23.8%

Analysis of Variance

Source             DF          SS          MS          F        P
Regression          3      2359.6       786.5       7.15    0.000
Residual Error     56      6163.3       110.1
Total              59      8522.8
```

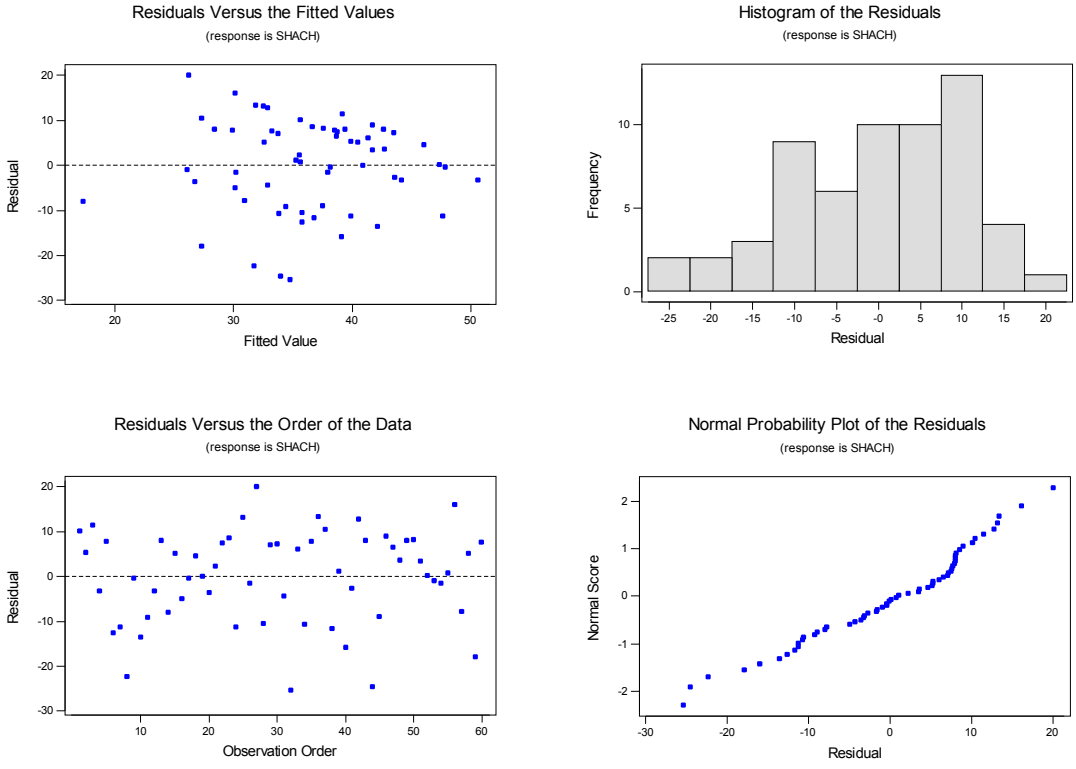

Figure 7 Residuals in relation to fitted value (top left); frequency distribution of the residuals (top right); residuals in relation to observation order (bottom left) and normal probability of residuals of the multiple regression analysis of survival on β-Hydroxy-acyle-coA-dehydrogenase, lactate dehydrogenase and pyruvate kinase activity

The straight line model is appropriate since no bowls are observed in the fitted vs residuals graph. The residuals are not homogeneous since a reduction of variance can be observed as the fitted value increase. The normality of the residuals is acceptable but not centered on zero. The assumptions for the use of a theroetical distribution are not met and an empirical distribution is proposed in order to test the significance of the parameters tested.

Randomization procedure

Randomization procedure was conducted in Systat 10.2 using the bootstrap procedure. The procedure used is the simplest of the randomization method offered by Systat 10.2. The commands used are as follows:

```
1  MODEL SHACH = CONSTANT+PK+HOAD+LDH
2  SAVE BOOT.SYD / COEF
3  EST /SAMPLE=BOOT(100000,60)
```

The first command line describes the model (in the GLM module) in the usual way. The second line ask Systat to save a file called "BOOT.SYD" containing the coefficient of each predictor. The third line tells Systat that the estimates will be realized on a bootstrapped sample containing 60 lines (the actual size of the sample). The number of iterations was set to 100 000 (why not?). The partial regression coefficients (slopes) of the regression our results were then compared to the empirical distribution generated (figure 8).

Table 2

Predictor	Coef	SE Coef	T	P(1)	P(2)
Constant	31.51	10.87	2.90	0.005	
PK	-4.251	1.208	-3.52	0.001	0.055
HOAD	37.627	8.981	4.19	0.000	<0.001
LDH	-4.164	1.192	-3.49	0.001	<0.001

P(1) = Probability calculated from theoretical distribution
P(2) = Probability calculated from the empirical distribution

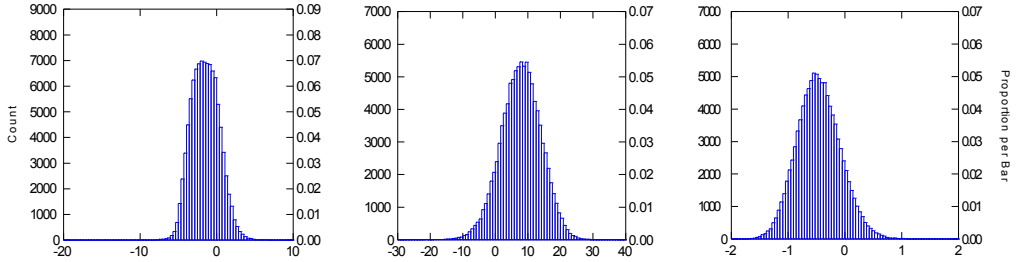

Figure 8 Distribution of the slopes (n = 100 000) of PK, HOAD and LDH generated by the randomization procedure.

The results are presented in the table 2. The use randomization changed the P-value in a way that the

slope of PK is no longer significantly different from zero (p = 0.055, table 2). However, PK activity will be kept in the model since the square multiple R decreased markedely when its effect was removed (not shown).

Discussion

During this experiment on egg quality, it has been impossible to relate eggs survival to a single enzymatic indicator, as presented in section II. Nevertheless, when more than one enzyme are used, a significant regression was found (SHACH = 31.5 - 4.16 LDH - 4.25 PK + 37.6 HOAD). Both anaerobic and aerobic glycolytic enzymes (LDH and PK) partial regression coefficients are negative, indicating that a high glycolytic activity can be related to poor egg quality. Interestingly, this study is one of the first to report anaerobic glycolysis activity during embryogenesis. Pyruvate kinase is a key enzyme of glycolysis and catalyze a reaction transforming phospho(enol)pyruvate into pyruvate; the last reaction of glycolysis. Newly formed pyruvate can then be integrated into the tricarboxylic cycle (Krebs cycle) or be anaerobically reduced to lactic acid by lactate dehydrogenase. As mentioned in part III, the partial regression coefficient of pyruvate kinase was not significantly different form 0 (P = 0.055) thus being less important in the relation. Also, a positive partial regression coefficient was obtained for HOAD, a key enzyme of lipid oxidation pathway, a strictly aerobic and very energetically effective pathway. These results suggest that main energetic pathway (lipid oxidation or anaerobic glycolysis) used late during embryogenesis influence or is influenced by egg quality. Surprisingly protein metabolism doesn't seem to be limiting since no relation has been found between survival and protein digestion (trypsin) and oxidation (AAT).

The weakness of the relation (R^2 = 0.238) between egg survival and metabolic enzymes found in this study suggests that enzymatic indicators are unlikely to be sensitive and precise indicators of egg quality. Attempts to relate egg quality on relative concentration of specific mRNAs (in progress) will be made during winter 2005. To date, the best predictor of survival at hatching is the proportion of eggs surviving from fertilization until eyed stage SHATCH = 0.765 SEYED – 11.468; R^2 = 0.753 (Lamarre, 2004b).

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