

## A multivariate analysis of condition of herring larvae from different environmental conditions

Hans Høie, Arild Folkvord and Arne Johannessen

University of Bergen

Department of Fisheries and Marine Biology

P.O. Box 7800, N-5020 Bergen

Norway

Correspondence to Hans Høie

E-mail: [Hans.Hoie@ifm.uib.no](mailto:Hans.Hoie@ifm.uib.no)

Fax: + 47 55584450

Tel: + 47 55584604

## ABSTRACT

Herring larvae were reared in laboratory at different temperatures (6 and 10°C) and different feeding levels from hatching and until eight weeks after hatching. Each larva was measured for developmental stage, standard length, dry weight, RNA, DNA and protein content. High survival, up to 92 % after eight weeks, was obtained. Larvae given high feeding levels (1200 prey per l) had exponential growth of dry weight, DNA and protein throughout the experiment. Almost no growth was observed in larvae given low feeding levels (40 prey per l) in the first half of the experiment, while an increase in the second half was seen. The RNA content of larvae given high feeding levels was not different at the two temperatures in spite of differences in somatic growth rates, which indicates lower RNA activity at lower temperatures. We found higher DNA concentrations in larvae reared at higher temperatures, which probably reflects temperature induces differences in growth through hyperplasia and hypertrophy. The measured variables of the larvae were used as input variables in a stepwise discriminant function analysis. The resulting classification functions were used to characterize the larvae from the different feeding regimes. The possibility of using differences in larval nucleic acid contents as a tool for discrimination of larval condition and regional origin is discussed.

## KEY WORDS

*Clupea harengus*, condition, development, larvae, multivariate analysis, temperature

## INTRODUCTION

The cause of the large fluctuations in recruitment of marine fishes is still not well understood, but the nutritional condition of larval fish has for a long time been considered to be of major importance (Hjort 1914). Several methods have been used to define larval condition over the years, but measurements at the cellular level like the use of nucleic acids are thought to be the most sensitive (Ferron and Leggett 1994). Nucleic acids play important roles in growth and development, where deoxyribonucleic acid (DNA) is the carrier of the genetic information and ribonucleic acid (RNA) is involved in protein synthesis and hence growth. The RNA/DNA ratio has been used to define larval nutritional condition since the DNA content is believed to represent cell number and remain constant regardless of nutritional status, while the amount of RNA is proportional to protein synthesis and thereby respond to starvation (Bergeron 1997). Growth and nutritional condition of fish larvae are strongly correlated (Clemmesen 1996) since most of the energetic food intake exceeding the metabolic cost is used for somatic growth. Growth of fish at the level of organism (whole fish) show intraspecific variation caused by environmental influence such as temperature and food supply (Weatherley 1990, Blaxter 1992), but can be estimated through the RNA/DNA ratio if the temperature is known (Buckley 1984). However, there are indications that both larval DNA and RNA content are influenced by temperature differently from somatic growth. Fish can grow by increasing the size of its muscle fibers (hypertrophy) or by recruitment of new muscle fibers (hyperplasia) (Goss 1966). The relative magnitude of these two processes is probably temperature dependent. Herring embryos incubated at higher temperatures have shown to have more numerous and smaller muscle fibers than larvae incubated at colder temperatures (Vieira and Johnston 1992, Johnston 1993). This response is most likely species specific since it contrasts with Atlantic salmon that had fewest but largest muscle fibres when incubated at the highest temperature (Usher *et al.* 1994). Varying concentrations of muscle fibres can be reflected in the estimated amount of DNA and thus also RNA/DNA ratio in a larva since hyperplasia often is associated with higher densities of nuclei (Nathanailides *et al.* 1994, Johnston *et al.* 1998).

Indications exist that the RNA concentration in fish is dependent on temperature through compensatory mechanism which reduce the amount of RNA needed to produce a certain amount of protein at higher temperatures (Goolish *et al.* 1984, Fergusson and Danzmann 1990). This effect is documented in adult fish but limited information on compensatory temperature effects is available for marine fish larvae.

The use of RNA/DNA ratio to estimate larval nutritional condition is restricted by lack of proper reference point. The ratio of well-fed larvae with high growth is dependent of ontogeny and varies between species. An interesting alternative to a certain RNA/DNA level as a reference point is to use multivariate approach as discriminant functional analysis to characterize the growth and condition of larval fish. Several measurements of larvae can then be used as input in a model. Discriminant functional analyses based on scales have successfully been used to separate groups of fish (Unwin and Lucas 1993, Barros and Holst 1995). Multivariate analysis of morphometry has also been used for herring larvae (McGurk 1985).

The nucleic acid content of herring larvae at some temperatures has been described earlier (Clemmesen 1987, Clemmesen 1994, Folkvord *et al.* 1996, Suneetha *et al.* 1999) but a complete range of temperatures that herring larvae might experience in nature are lacking. Data on herring larvae larger

than 25 mm is also scarce. We therefore performed an experiment where herring larvae were reared at different temperatures and feeding levels, which resulted in large differences in size at age between the groups. The dry weight, standard length, RNA, DNA and protein content were measured on each individual larva. We performed discriminant functional analysis to evaluate the potential of this method in separating groups of larvae.

## MATERIALS AND METHODS

Eggs from Norwegian spring spawning herring were incubated at  $6.0^{\circ}\text{C} \pm 0.3$  (mean  $\pm$  S.D.) in running filtered seawater under natural photoperiod conditions. 600 larvae were transferred to each of eight 500 l green tanks. The tanks had stagnant seawater and perforated tubes for aeration at the bottom supported water mixing and were covered with 3 mm grey Plexiglass lids. The larvae were reared at two different temperatures (6 and  $10^{\circ}\text{C}$ ) and two nominal food densities (low = 40 and high = 1200 prey per litre) with two replicates, resulting in eight tanks. Larvae given high food densities at 10 and  $6^{\circ}\text{C}$  are named the 10H and 6H group respectively, and larvae given low food densities at 10 and  $6^{\circ}\text{C}$  are named the 10L and 6L group. Natural photoperiod for the time of the year and latitude was maintained through the experiment by a computer programme Lysstyr 2.0 (Hansen 1990). On day 3 after hatching phytoplankton (*Rhodomonas* sp. and *Isochrysis* sp.) was added to each tank. On day 4 natural zooplankton were added in predetermined concentrations to the respective tanks. The zooplankton prey for the herring larvae were collected from the seawater pumped from 3 meters depth and filtered through a UNIK filter. Prey levels were adjusted every day (in the morning) to the predetermined level given for each individual tank. The temperature in the tanks was measured each morning, while oxygen concentrations and salinity were measured once a week. The experiment was terminated on day 56 after hatching. Twenty larvae were sampled in each tank every seventh day from hatching to day 56 after hatching except for the 10L tanks that were terminated on day 42. Biochemical analyses were carried out on 10 larvae from the different groups and sampling dates from day 7 to day 49. Initial samples of 12 larvae were used for newly hatched larvae (day 0). Overall survival in each tank was estimated by assigning the same daily mortality rate to the sampled larvae as the larvae remaining in the tank in the end of the experiment.

Sampled larvae were measured for standard length (SL) alive and staged according to (Doyle 1977) with the inclusion of an extra stage 1d representing larvae with no visible yolk remains or dorsal fin structures (Øiestad 1983). Prior to RNA and DNA analyses the larvae were dried in a freeze-dryer for 18-24 hours, thereafter transferred to a desiccator, and weighed on a Sartorius microbalance to the nearest  $\mu\text{g}$ . The RNA and DNA analyses were measured by the method described by Suneetha *et al.* (Suneetha *et al.* 1999), but with 0.01 % sodium dodecylsulfate in the Tris EDTA buffer. Total protein content of the larvae was also estimated. Ten  $\mu\text{l}$  of the supernatant was transferred in triplicate to a microplate and kept frozen at  $30^{\circ}\text{C}$  until analyses. Protein concentrations were determined photometrically with a Titertek Multiscan MCC (540 nm filter) by Pierce BCA protein assay reagent method.

Mean daily growth rate ( $\text{mm day}^{-1}$ ) of standard length was calculated as  $(L_2 - L_1) / (t_2 - t_1)$  where L is mean standard length at time  $t_2$  and  $t_1$ . Specific growth rate, SGR, ( $\% \text{ day}^{-1}$ ) was calculated using the equation  $(e^G - 1) * 100$  where  $G = \ln(W_2) - \ln(W_1) / (t_2 - t_1)$  and W was mean dry weight, DNA, RNA or protein content at time  $t_1$  and  $t_2$ .

Analysis of covariance (ANCOVA) was used on log transformed values when the group specific trends could be described by a linear relationship. A common polynomial equation was fitted when a curvilinear function best described the relationship between two variables (Kleibbaum *et al.* 1988). Differences between groups were then examined by testing the residuals by a Student t-test. Principal component analysis were used to group correlated variables (Kleibbaum *et al.* 1988). Input in the analyses were larval stage, SL, RNA/DNA ratio, log-transformed values of protein, DW, RNA and DNA, and residuals of the group specific linear regression of  $\text{LnDW}$  versus  $\text{LnSL}$  and  $\text{LnRNA}$  versus  $\text{LnDNA}$ . Forward stepwise discriminant function analyses (Kleibbaum *et al.* 1988) was performed on log transformed values (except for SL) in order to test if the morphometric and biochemical variables could be used to separate larvae with different feeding and temperature histories. The discriminant function were developed by using one of the parallels, and the classification functions were then tested on the other parallels. Yolk sac larvae (developmental stages 1a – 1c) were excluded from this analysis since they were partly independent of the feeding treatment. All the data presentations and statistical analysis were carried out using Statistica® (version 5.5, 99 edition) for Windows.

## RESULTS

Survival of the larvae at the termination of the experiment (day 56) ranged from 50.2 to 92.2 % (Table 1) with highest values of larvae in the 6H group, and lowest values of larvae in the 10L group. The

parallels showed almost similar values. The temperature was kept close to the predetermined values, but the prey levels showed some variations (Table 1).

Growth in standard length (SL) was highest at 10°C and at the highest prey densities (Figure 1). A temperature effect with highest growth at 10°C was evident at high prey densities but not at low prey densities where both temperature groups had minimal growth up to day 35 and a little increase afterwards. Larval dry weight (DW), DNA, RNA and protein content increased linearly on a log-scale with age in the same manner as SL in the 10H and 6H groups. Daily SGR of DW from day 7, when the yolk was depleted, to day 49 was 9.2 % and 4.4 % in the 10H and 6H groups respectively (Table 2). The 10L and 6L groups had SGR of DW of 1.9 % and 1.7 % in the same period at 10 and 6°C respectively. Larvae offered low prey densities had reduced, but positive growth rates from day 7 to termination of the experiment (Table 2). However, the larvae given low prey densities didn't grow much from day 7 to day 35 after hatching. No significant relationship between larval DW or protein content versus age were found at 6 and 10°C, or between larval DNA content at 10°C versus age in that time period (linear regression,  $p > 0.05$ ). RNA content versus age at both temperatures and DNA content versus age at 6°C had negative slopes, while SL versus age had positive slopes at both temperatures in the same time period (linear regressions,  $p < 0.01$ ).

Larvae reared at high prey densities were also heavier, longer and contained more DNA at given developmental stages after yolk depletion than larvae reared at low prey densities. There were also differences in larval DNA content between temperature groups of larvae given high prey densities. Larvae reared at 10°C contained more DNA than those reared at 6°C at developmental stages 2b, 2c and 3a (t-tests,  $p < 0.05$ ).

Larval RNA and DNA content were analyzed by ANCOVA where larvae DW was used as a covariate. Only larvae with SL < 22 mm were used since we wanted to compare larvae reared at different temperatures but in the same size range. Larvae reared at 10°C contained 9.4 and 8.3 % more DNA than at 6°C at high and low prey densities respectively (ANCOVA,  $P < 0.001$ , Figure 2). No differences between temperature groups among larvae at both prey levels were found on larval RNA content (ANOCVA,  $P > 0.05$ ). When protein content was set as covariate instead of DW the same general pattern was seen for nucleic acids content. Larval protein content was not significantly different between the two temperature groups neither at low nor high prey densities (ANCOVA,  $P > 0.05$ , DW as covariate). RNA content of larvae given high prey densities was 16.5 % higher at 6 °C compared to 10°C when DNA was set as covariate (ANCOVA,  $P < 0.001$ ), but there was a small curvilinear trend at 6°C. T-test of common second order residuals of LnRNA versus LnDNA of larvae given low prey densities showed that also larvae reared at 6°C had more RNA than those reared at 10°C.

Larvae reared at the same prey levels but different temperatures showed more similar pattern in factor loading plots of the principal component analysis than larvae reared at the same temperature but at different prey densities (Figure 3 and 4). Factor 1, which can be interpreted as a size factor explained 65 – 73 % of the total variance in larval groups reared at low and high prey densities respectively. Note that the RNA/DNA ratio of the larvae fed *ad libitum* show some correlation with larvae size. Factor 2 seems to represent the biochemical condition measures (the RNA/DNA ratio and residuals of LnRNA versus LnDNA content) for larvae given high prey densities while factor 3 represents the morphometric condition measure (the residuals of LnDW versus LnSL). Note the lack of correlation between the morphometric and biochemical condition indices and that the RNA/DNA ratios are more correlated to the size factor than the condition indices based on residuals. The four larval size measurements (SL, DW DNA and protein content) are very closely associated to larvae given high prey densities, but SL differ somewhat from the other and are more related to larval stage in the groups given low prey densities. Some differences were seen between the two temperature groups of larvae given low prey densities. At 10°C factor 2 seems to represent both condition indices based on residuals, while the residuals of LnRNA on LnDNA was less correlated with the residuals of LnDW on LnSL at 6°C.

Three variables were included in the discriminant function analysis; LnRNA, LnDNA and SL. The model correctly classified 68 – 90 % of the larvae that were used to derive the model (Table 3). Most of the discrimination of the 10H group was provided at the first axis, while the 10L and 6H were discriminated by the second axis (Figure 5). The correct classifications of larvae not used to derive the model were high for all groups (87.5 – 100 %), except for larvae from group 6L where 41.7 % were correctly classified (Table 3). Only 30 % of the 6L larvae older than 35 days, when higher growth rates occurred, were correctly classified. About 60 % of the larvae were then classified as belonging to the 6H group. Most of the incorrect classification of larvae of age 35 days and younger was to the 10L group.

## DISCUSSION

Temperature was found to have effect on larval growth rate, developmental rate and nuclei acid content at given larval size in this study. However, the temperature effect was dependent of feeding level. Larvae reared at low feeding level reached each developmental stage after yolk absorption at a smaller somatic size than larvae reared at high feeding level. Similar result has been found on carp larvae where quality and quantity of food influenced on the timing of developmental steps (Kamler *et al.* 1990). An uncoupling of organogenesis at different temperatures during the egg stage have been found in herring and other species (Fukuhara 1990, Johnston 1993), and the size at which ontogenetic events of larvae occur can be influenced by temperature (Chambers and Leggett 1987). However, the general picture that elevated temperatures appear to accelerate the rate of ontogeny more than the rate of growth (Fuiman *et al.* 1998) is not supported by our data on herring larvae. Larvae given high food densities had higher growth rates at 10°C than at 6°C while only very small differences in larval size at each developmental stage were found.

We found some indications that compensatory mechanism can influence larval RNA content. No temperature difference on RNA content was found on larvae fed at both prey levels. However, larvae given high food rations and reared at high temperature had higher growth rates than larvae reared at low temperatures. Thus, it seems that herring larvae need more RNA to maintain growth at a lower temperature, which probably reflects lower RNA activity (Goolish *et al.* 1984, Miglavs and Jobling 1989).

We found that larvae reared at 10°C had higher DNA content than similar sized larvae reared at 6°C at both feeding levels. This is most likely caused by differences in muscle fibres size and nuclei densities. Johnston *et al.* (1998) found that herring larvae reared at higher temperatures had more muscle fibres per myotome and higher densities of nuclei than similar sized larvae reared at lower temperatures. They concluded that the relative contribution of hypertrophy (increasing the size of the muscle fibres) and hyperplasia (recruitment of new muscle fibres) to growth varies with temperature. Temperature have also been found to influence muscle fibre size in many other species (Usher *et al.* 1994, Koumans and Akster 1995, Galloway *et al.* 1998). The two types of growth mechanisms are therefore important to consider when using nucleic acids to estimate nutritional condition and growth. There are also indications of individual variations in hyperplasia that can explain some of the variation in estimated DNA content between larvae (Koumans *et al.* 1993). The recruitment of new muscle fibres pauses at the time of hatching, but continues at 15 mm total length in herring (Johnston *et al.* 1998). In general, recruitment of new muscle fibres in fish will not cease until the fish reach approximately 44 % of maximum length (Weatherley 1990). The dynamics behind the increase of muscle fibre nuclei in growing fish is complex. Embryonic muscle fibres arise from fusion of several myoblast to form multinucleated muscle fibres. The myosatellite cells proliferate to produce new myonuclei that are necessary for muscle growth (Koumans and Akster 1995). Different subpopulations of myosatellite may also be involved in hyperplasia and hypertrophy (Koumans *et al.* 1993). The measured DNA of fish larvae consists therefore of both muscle fibre nuclei and myonuclei.

Many different tools and techniques have been used in order to separate different fish stocks and sub-units within fish stocks like allele frequencies, vertebrae number, scale size, otolith structures and otolith chemistry (Parsons 1972, Jørstad *et al.* 1991, Moksness and Fossum 1991, Barros and Holst 1995, Kinnison *et al.* 1998, Campana 1999). This study suggest that larval nucleic acid and larval length, three easily measured traits, also have a potential to discriminate larvae that have experienced different environmental conditions. The inclusion of larval DNA content in the discriminant function probably reflects the different degree of hyperplasia and hypertrophy between groups. In addition to temperature, larval growth rate is also known to influence muscle development with decreased contribution of hyperplasia to muscle growth in slow-growing larvae (Koumans *et al.* 1993, Alami-Durante *et al.* 1997, Galloway *et al.* 1999). The classification function separated well larvae with different growth rate (i.e. given different feeding regimes) reared at 10 °C in this study. The slow-growing larvae of the 6L group had the lowest percent correct classification which means that their properties were less unique than for the other groups. Its also interesting to note that larval length and not dry weight or protein content was included in the discriminant function. Factor loading plots of principal component analysis also show that larval length is less grouped with dry weight and protein content, but is more related to larval development than the other measurements of larval size. Fuiman *et al.* (1998) also concluded that SL and LogSL approximated the timing of ontogenetic events of fish larvae with minimal variability, while LogDW was a poor metric for ontogenetic state. The larvae we used to derive the discriminant function were given different treatments from hatching and throughout the larval period. Early thermal experience can produce irreversible changes in phenotype of fish (Blaxter 1992). There are also indications that incubation temperature can have long time effects on muscle development (Nathanailides *et al.* 1994). Johnston *et al.* (1998) found that incubation

temperature had small effect on growth through hypertrophy and hyperplasia at hatching and first feeding, but effects of incubation temperature could be found 82 days after hatching. They suggested that incubation temperature influenced the number of stem cells and/or the number and properties of the numerous cell types involved in growth regulation. The determination of the amount of hyperplasia is also suggested to be of genetic influence in herring. Green Walker et al. (1972) showed that the total fibre number in adult herring is stock-specific.

The application of a method utilizing temperature-induced differences in larval muscle traits as a tool for discrimination of larval condition and regional origin must be based on proper reference material since there are interspecies variation in the temperature response (Alami-Durante *et al.* 1997, Galloway *et al.* 1999). The assumption of higher amounts of larval DNA at periods of high hyperplasia also need closer examination. High amounts of DNA were found in carp at time of high growth through hyperplasia but the DNA concentration decreased relatively faster than the decrease of hyperplasia at increasing fish length (Koumans *et al.* 1993).

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Table 1. Hydrographic data and survival percentage in the different tanks. Number in brackets are one standard deviation. Number after tank names refer to parallel 1 and 2.

Tank	Temperature (°C)	Salinity (‰)	Oxygen saturation (%)	Mean prey levels (l <sup>-1</sup> )	Survival %	Daily mortality rate (%)
6H1	5.9 (0.2)	33.7 (0.1)	95.3 (1.7)	1158.8 (150.0)	90.2	0.18
6L1	6.2 (0.1)	33.8 (0.1)	97.3 (2.3)	30.9 (11.0)	65.4	0.75
6H2	6.1 (0.2)	33.5 (0.1)	96.3 (2.0)	1112.7 (150.0)	92.2	0.14
6L2	6.2 (0.2)	33.7 (0.1)	96.4 (2.2)	30.0 (8.5)	63.9	0.80
10H1	9.9 (0.3)	33.4 (0.3)	97.7 (2.2)	940.6 (145.1)	79.1	0.42
10L1	10.1 (0.2)	33.8 (0.2)	97.1 (0.9)	25.7 (8.0)	50.5	1.21
10H2	10.1 (0.1)	33.5 (0.4)	95.0 (2.9)	883.1 (143.2)	83.8	0.31
10L2	10.1 (0.1)	33.7 (0.1)	96.9 (1.5)	26.9 (10.1)	50.2	1.22

Table 2. Growth rates of the larvae. Paralleles are pooled.

Group	Age range (days)	Standard length (mm day <sup>-1</sup> )	Dry weight (% day <sup>-1</sup> )	Growth rate		
				DNA (% day <sup>-1</sup> )	RNA (% day <sup>-1</sup> )	Protein (% day <sup>-1</sup> )
6H	7 – 49	0.24	4.4	3.1	4.5	4.2
6L	7 – 49	0.10	1.7	1.7	1.6	1.6
10H	7 – 49	0.40	9.2	7.8	8.4	8.4
10L	7 – 42	0.08	1.9	1.0	1.5	1.7

Mean daily growth rate (mm day<sup>-1</sup>) of standard length was calculated as  $(L_2 - L_1) / (t_2 - t_1)$  where L is mean standard length at time  $t_2$  and  $t_1$ .

Specific growth rate (% day<sup>-1</sup>) was calculated using the equation  $(e^G - 1) * 100$  where  $G = \ln(W_2) - \ln(W_1) / (t_2 - t_1)$  and W was mean dry weight, DNA, RNA or protein content at time  $t_1$  and  $t_2$ .

Table 3. Classification matrix of the larvae used to derive the discriminant function, and for larvae in the parallel.

True group	Predicted group for larvae used to derive the function				Predicted group for the parallel			
	6 H	6 L	10 H	10 L	6 H	6 L	10 H	10 L
6H	27	5	2	2	27	8	3	0
6L	1	17	0	1	0	10	0	2
10H	0	0	27	1	0	0	27	1
10L	3	2	0	22	0	6	0	21
Percent correct	90.0	68.0	93.1	84.6	100.0	41.7	90.0	87.5

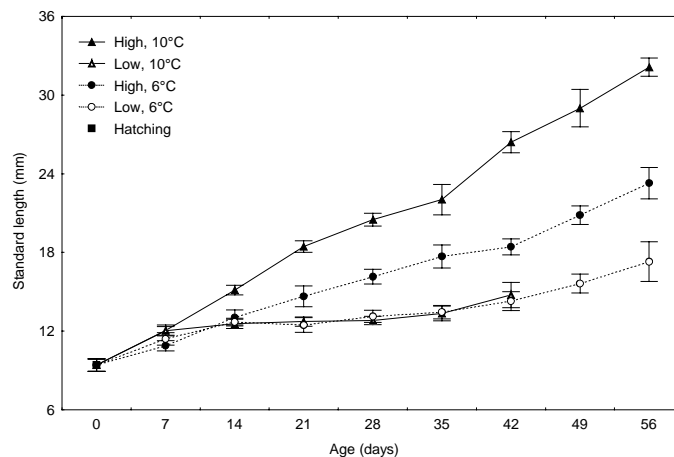


Figure 1. Larval standard length from hatching to age 56. High and low refers to nominal prey densities of 1200 and 40 per litre respectively. Means  $\pm$  2 SE are given.



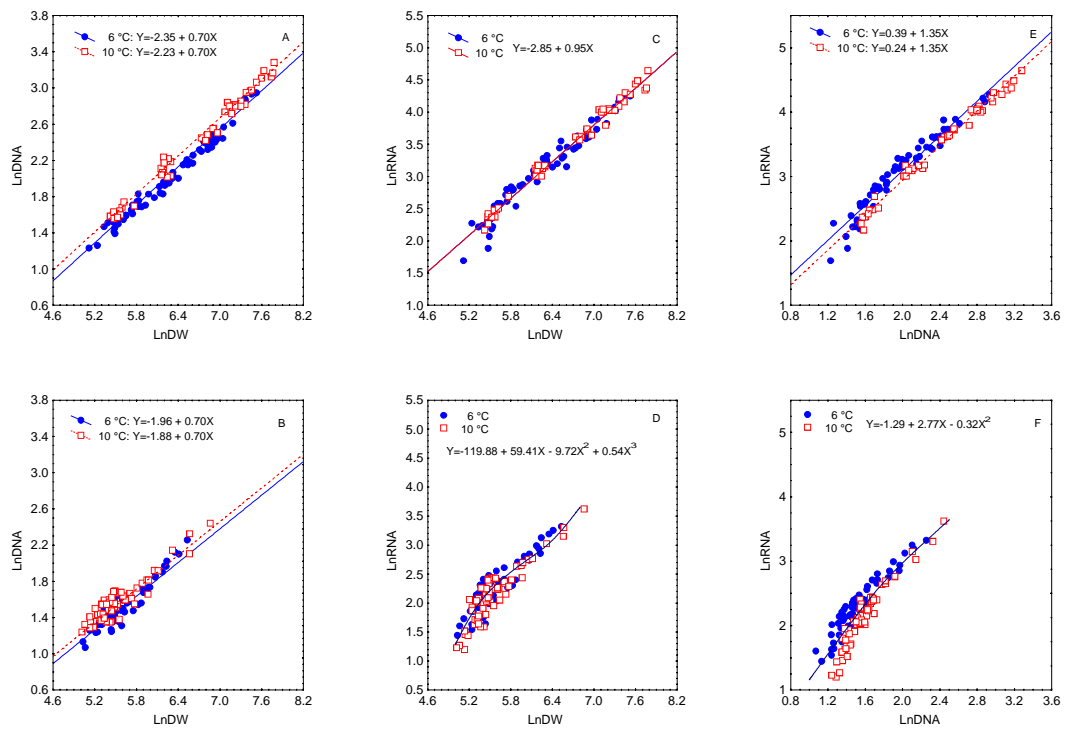


Figure 2. Relations between larval RNA and DNA content versus DW and DNA content at 6 and 10°C. The equations in A, B, C and E are derived from ANCOVA analyses, while the equation in D and F are best fitted polynomial equations. A, C and D are larvae given 1200 prey/l and B, D and F are larvae given 40 prey/l.

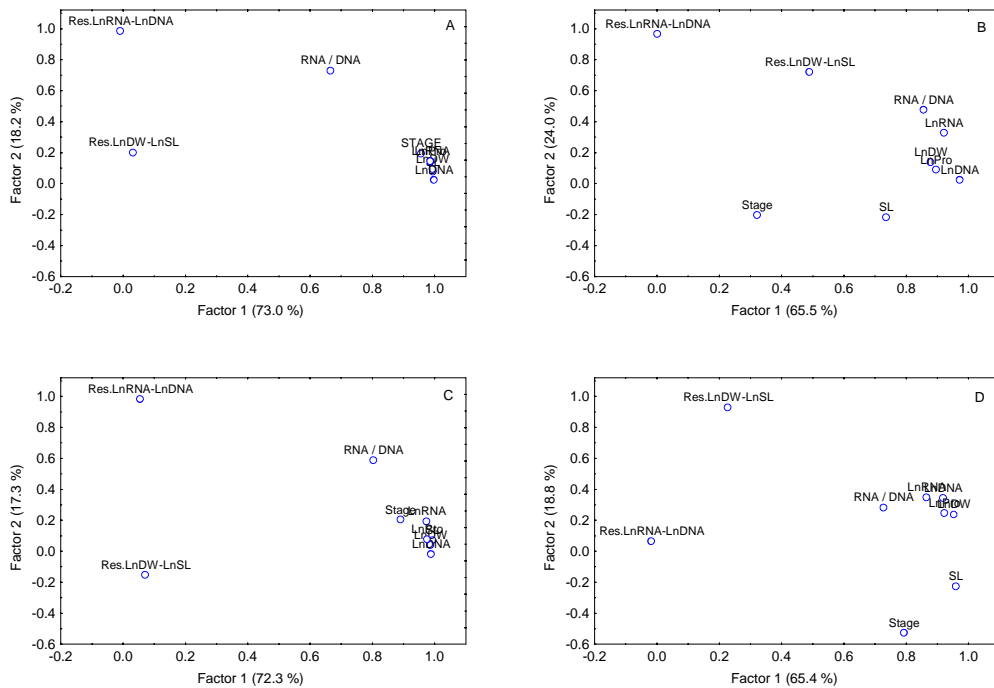


Figure 3. Factor loading plots with varimax normalized rotation showing factor 1 and 2. Larval stage, SL, LnDW, LnRNA, LnDNA, LnProtein (LnPRO), RNA/DNA ratio and residuals of LnRNA versus LnDNA and LnDW versus LnSL are input variables. A: 10°C and 1200 prey/l, B: 10°C and 40 prey/l, C: 6°C and 1200 prey/l, and D: 6°C and 40 prey/l.

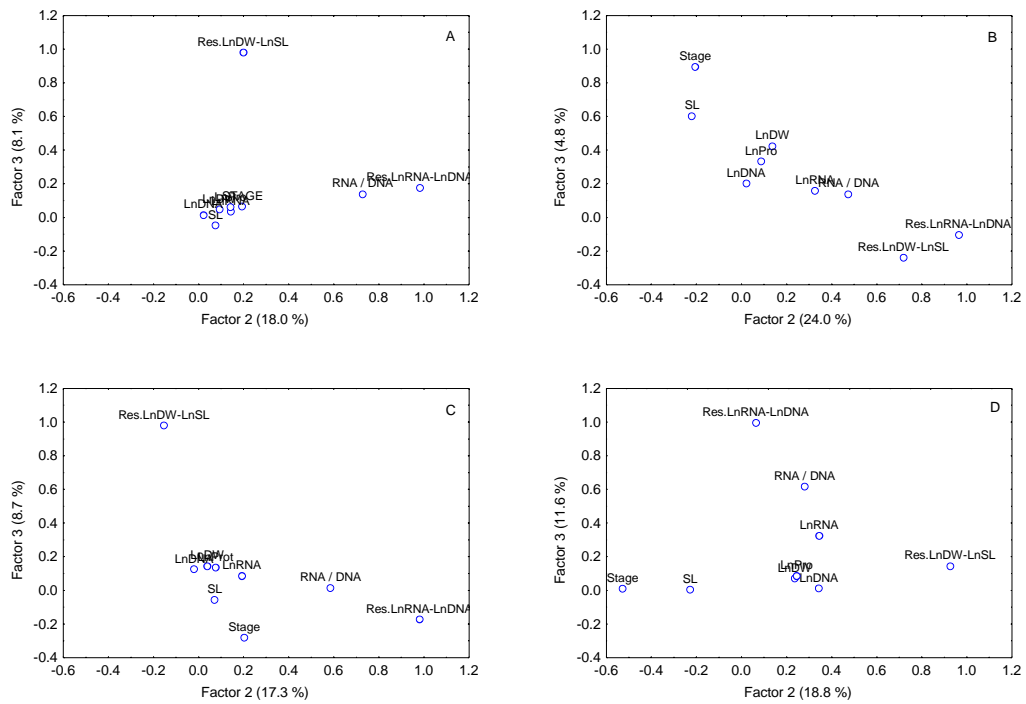


Figure 4. Factor loading plots with varimax normalized rotation showing factor 2 and 3. Larval stage, SL, LnDW, LnRNA, LnDNA, LnProtein (LnPRO) and RNA/DNA ratio and residuals of LnRNA versus LnDNA and LnDW versus LnSL are input variables. A. 10°C and 1200 prey/l, B: 10°C and 40 prey/l, C: 6°C and 1200 prey/l, and D: 6°C and 40 prey/l.

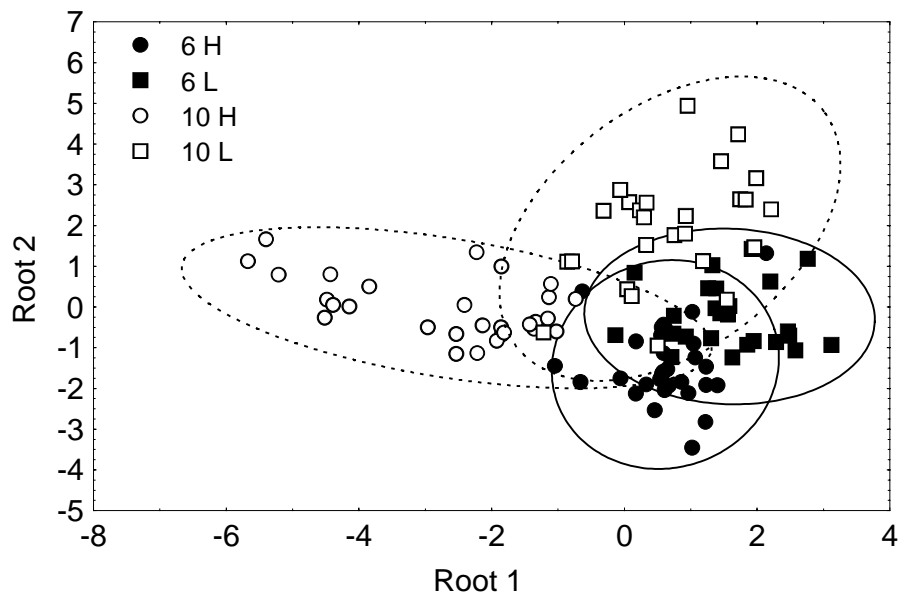


Figure 5. Scatterplot of canonical scores for the four larval groups.