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EVALUATING THE ROLE OF ENDOGENOUS AND EXOGENOUS FEATURES ON LARVAL HAKE NUTRITIONAL CONDITION

Running title: Nutritional condition of *Merluccius hubbsi* larvae

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ABSTRACT AND KEYWORDS

Evaluating the nutritional condition of fish larvae is imperative to establish the importance of starvation, understanding the causes of larval mortality and determining favorable zones for growth and survival within nursery areas. We assessed the nutritional condition of *Merluccius hubbsi* larvae employing RNA/DNA index (*sRD)* and its derived index of growth performance (*Gpf*). Larvae (N=395) were collected during the reproductive peak in 2010 and 2011. Principal Component Analysis and Generalized Linear Models were used to study the relationship between larval condition and endogenous factors (size, weight, growth rate, trophic incidence and carbon content per stomach) and exogenous variables (temperature, chlorophyll concentration, potential prey availability, ctenophore biomass, and hake larvae density). The year of sampling was also considered as a variable. The significant variables in the model selection were size, temperature, the density of hake larvae, and year. The larval size was positively related to the condition while larval density and temperature showed a negative relationship with the *sRD* index. The year was also a significant variable, with higher larval *sRD* values in 2010. The negative relationship between *sRD* and larval density suggests the existence of mechanisms of density-dependence operating upon larval condition. On the other hand, the lower temperatures occurred in stratified waters (with greater availability of food) a fact that might explain the negative relationship between *sRD* and temperature. The fitting of the models

suggests that other explanatory variables might be considered to improve the understanding of the nutritional condition index nature.

Hake, larva, RNA/DNA, growth, generalized linear model, principal component analysis, Atlantic

1. INTRODUCTION

Variability in fish recruitment is a consequence of various processes that operate at different spatial and temporal scales. To understand this variability, it is necessary to study the factors that determine the survival of the early developmental stages of fish. These factors can be both physical and trophodynamic and act throughout the pre-recruit life of fish (Houde, 2008).

Nutritional condition assessment allows evaluating the individual physiological state of larvae, which is an echo of the environmental situation to which they have been exposed. The RNA/DNA (*RD*) index is the most used biochemical index as an indicator of the nutritional status of fish larvae (Ma et al., 2014; Foley et al., 2016). The *RD* index measures the cellular biosynthetic capacity and correlates with the larval nutritional status (Ferron and Leggett, 1994; Buckley et al., 1999).

The common hake, *Merluccius hubbsi,* constitutes one of the most important fishing resources of the Argentine bottom trawling fleet, with a total catch of approximately 310,000 tons reported in 2019 (Ministerio de Agroindustria, 2019[1]). It inhabits waters of the South West Atlantic Ocean between Cabo Frio in Brazil (22°S) and southern Argentina (55°S), in depths ranging between 50 and 500 m (Cousseau and Perrota, 1998). There are two fishing stocks, located north and south of 41°S, respectively. The Southern or Patagonian stock, which will be the object of the present study, is the most abundant population of this species and represents 85% of its total biomass (Aubone et al., 2000). During the late 1990s, the spawning biomass of both hake stocks decreased drastically, which was attributed mainly to the increase in fishing pressure on this resource during the previous years (Aubone et al., 2000). The reproductive season of the Patagonian stock takes place on the North Patagonian shelf, in the vicinity of Isla Escondida (43°30'-44°S and 65°W), mainly between November and April with a maximum spawning in December and January (Macchi et al., 2004; 2007). But in recent years, changes in the parental stock structure and the location of the spawning females have been observed (Macchi et al., 2010).

During austral summer the north Patagonian shelf is characterized by a frontal structure that separates homogeneous coastal waters from stratified ones located towards the open sea (Glorioso, 1987). This frontal system is characterized by high availability of nitrates that favors biological productivity, generating significant blooms of phytoplankton (Carreto and Benavidez, 1990) and large aggregations of copepods (Derisio et al., 2014; Temperoni et al., 2014). Also, large aggregations of gelatinous zooplankton have been recorded in the tidal front area (Mianzan and Guerrero, 2000). The Península Valdés tidal front is important to several fish resources since it creates a diversity of suitable spawning habitats for the adults, and breeding areas for their eggs and larvae (Alemany et al., 2014). Bakun and Parrish (1991) characterized this region as a successful area for fish reproduction since it would fulfill the "fundamental triad" hypothesis (Bakun, 1996). The combination of nutrient enrichment, food concentration, and retention of larvae should improve the survival of hake's early developmental stages in this area. Retention of hake larvae in this frontal area has been previously observed, a fact that was attributed to the coupling of larval behavioral patterns and water circulation (Álvarez-Colombo et al., 2011). Early hake larvae, smaller than 4 mm and younger than seven days, were grouped near the thermocline. On the other hand, older larvae (able to perform daily vertical migrations) remained near the bottom during the day and at the thermocline during the night (Álvarez-Colombo et al., 2011).

Assessment of nutritional condition represents a useful instrument to determine favorable breeding areas, provides tools to explain variability in the species recruitment, and can be used for the integral management of a fishing resource of great importance as is the case of *M. hubbsi*. This work constitutes a comprehensive analysis of the factors that influence the nutritional status of hake larvae in the Patagonian stock of the species. We considered endogenous variables (larval size, developmental stage, age, weight, otolith recent growth rate, trophic incidence) as well as exogenous variables (temperature, chlorophyll *a* concentration, availability of potential prey, gelatinous zooplankton biomass, and hake larvae density) as potential explanatory variables. This study aims to explore which are the driving factors for *M. hubbsi* larval nutritional condition that therefore will affect larval survival and their posterior recruitment to adult stages. Finally, because the response of fish larvae nutritional condition to environmental drivers strongly depends on ontogeny, we accounted

for this variability by comparing the larval condition of the two consecutive sampling years, taking into account larval developmental stage.

2. MATERIALS AND METHODS

2.1. Sample collection

The material used in this work came from two research surveys, conducted by the Instituto Nacional de Investigación y Desarrollo Pesquero (INIDEP), for the purpose of estimating the abundance of spawning individuals and early life stages of southern hake stock during its reproductive peak in January of 2010 and 2011. Ichthyoplankton and gelatinous zooplankton samples were collected with a Bongo net (diameter 60 cm; meshes of 300 and 500 μm) equipped with flowmeters in the mouth (to calculate the volume of filtered water in each tow) and a SCANMAR depth sensor. The net was operated at an average speed of 2.5 knots by oblique trawls above the bottom (from 2 to 10 m above it), up to the surface. For the 2010 and 2011 surveys, 45 and 28 fixed-site ichthyoplankton stations were sampled respectively. In each survey, additional hauls were made to validate hake larvae acoustic signals. In these cases, horizontal trawls coinciding with the depths of the acoustic marks were carried out with a Rectangular Midwater Trawl (RMT, mouth 1 m^2; 500 μm mesh) when the eco-records were far from the sea bottom, and an Epibenthic Sampler (MEB, mouth 0.36 m^2; 1000 μm mesh), when they were close to the bottom. The speed at which these nets were operated was of 3 and 2.5 knots, correspondingly. In Figure 1 and Table 1, the position and details of sampling where larvae were collected are presented. Larvae utilized for biochemical studies were separated on board, immediately fixed in liquid nitrogen, and subsequently stored in a freezer at -80°C until processing. The remaining ichthyoplankton sample was fixed with 5% formaldehyde in seawater for larval quantification in the lab. At each station salinity and temperature data were recorded at different depths using a Seabird 911 CTD probe.

Water samples were collected at all stations from the surface with Niskin bottles to determine chlorophyll *a* (*Chla*) concentration as an indicator of phytoplankton biomass. The water samples were filtered through glass fiber filters GF/F (0.7 μm pore size) at low luminosity using a low-pressure

vacuum pump and then stored in liquid nitrogen on board. *Chla* determination was carried out using the fluorometric method of Holm-Hansen et al. (1965) with modifications (Lutz et al., 2010).
To quantify the potential food for hake larvae, zooplankton samples were collected (Figure 1A) using a Minibongo net (diameter 18 cm; mesh of 67 μm) equipped with a flowmeter and depth sensor. Oblique hauls were performed from bottom to surface and the zooplankton samples were preserved in 5% formaldehyde in seawater. At the laboratory, the abundance of copepod adults and developmental stages smaller than 2 mm was determined using the calculation of the filtered water volume obtained through the flowmeter and expressed as the number of individuals per cubic meter.

2.2. Larval sample processing

Larvae were thawed and photographed in the laboratory and their standard length (*SL*) measured with a micrometer attached to a Zeiss dissecting microscope with an Axio-Vision software. Yolk sac larvae were not used in this study. Previous studies on *M. hubbsi* larvae shrinkage caused by freezing fixation revealed an important variability on differences between pre and post-fixation measurements; therefore we decided not considering the shrinkage effects (Diaz et al., 2015). Larvae collected in both cruises were stored for a similar length of time prior to analysis. Hake larvae were classified under magnifying glass according to the notochord flexion stage and hypural development as specimens in Preflexion (3.0-6.49 mm *SL*), Flexion (6.5-7.99 mm *SL*), Postflexion (8.0-18.0 mm *SL*) or Transformation (>18.0 mm *SL*) according to Betti et al. (2009) criteria. The head and digestive tract were dissected. The heads were preserved in 96% ethyl alcohol for further studies of age, and the digestive tubes in formalin 5% for stomach contents analysis. The muscle trunks were lyophilized and weighed to the microgram with a Sartorius microbalance (N=395).

2.3. Age and growth determinations

Under a dissecting microscope, the *sagitta* otoliths were extracted and mounted in a transparent medium and dried for 48 hours before the analysis of the microstructure. The optical microscope observation was made (1000 X) using immersion oil. The age of each individual was determined by counting the number of increments of daily growth along the axis of the greater radius of the otolith, from the core to the edge. A 90% coincidence in the number of increments was assured between the

two otoliths from the same individual, and one of them was then randomly selected for posterior analyzes. When only one otolith was available/undamaged, its number of increments was considered. Digital image processing software (Kontron program) was used for measuring the otolith increments according to Brown et al. (2008). The recent growth of the otoliths (*RGR*) was determined by measuring the thicknesses of the last three fully formed increments (Leonarduzzi et al., 2010). The mean width of the last fully formed increments for each organism was calculated (μm).

2.4. Stomach content analyses

Trophic incidence (*NpreyStom*) was calculated as the total number of prey items in each gut content (Hyslop, 1980). Prey species were identified to the lowest taxonomic level, and several measurements (in mm) were taken to estimate carbon content (mgC per stomach (*CperStom*). Such measurements included egg diameter, total length and width for nauplius stages, and prosome length and width for copepodid and adult stages. The biovolumes of copepod adults and copepodites were estimated using appropriate length and mass relationships available from the literature (Temperoni et al., 2013 and references therein). For eggs and nauplius stages, biovolume was estimated from geometrical shapes similar to their body configuration (Cass-Calay, 2003). Dry mass was obtained by multiplying the wet mass by 0.20 and the carbon content (mgC) was considered to be 40% of the dry weight (Postel et al., 2000).

2.5. Larval nutritional condition

The protocol used for the nutritional condition analysis was described by Caldarone et al. (2001), with the modifications made by Diaz and Pájaro (2012) to maximize the detection of nucleic acids in 1 ml cuvette instead of microplates. Nucleic acid extraction was performed by cell lysis from the muscular trunk of each larva using 1% Sarcosyl Tris EDTA and 1-hour agitation. Samples were then centrifuged for 15 min at 13400 rpm (14658 g). From the supernatant obtained from each larva, 100 μl were taken and brought to a volume of 1000 μl in Tris EDTA Buffer and Ethidium Bromide (0.1 mg ml^{-1}) was used as fluorochrome. Determinations were performed using a Perkin Elmer spectrofluorometer (Ex: 360 nm and Em: 590 nm). Two readings were recorded for each larva, the first one corresponding to the total nucleic acids and a second one after the addition of 50 μl of

RNAase enzyme (bovine pancreas RNAase A, Sigma R 6513, 20 U ml^{-1} and incubation for 30 min at 37°C) corresponding to the DNA content. Endogenous fluorescence (prior to the addition of Ethidium Bromide) was insignificant, so it was not considered. In accordance with previous studies using larvae from the same species (Diaz et al., 2014) we assumed that residual fluorescence was insignificant so DNAase enzyme was not used. The amount of RNA in each larva was determined by difference. On the same day of the analysis calibration curves of DNA (lambda DNA, digested with Hind III, Roche 10236250001) and RNA (16S-23S RNA from *Escherichia coli*, Roche 10206938001) were used to calculate nucleic acid concentration in the samples. The average value for the slope of the calibration curves obtained in the present study was 2.50 (± 0.33 SD). The RNA/DNA index (*RD*) was calculated individually and reported values corresponded to the muscle trunks. The *RD* index of 395 larvae of *Merluccius hubbsi* were determined, using 363 for the statistical analyses. The *RD* values obtained in this study were standardized (*sRD*) according to the procedure described by Caldarone et al. (2006). A growth rate (*G*) was estimated using the best fit model obtained by Buckley et al. (2008) developed for other gadoids (cod and haddock) that relates temperature (*T*) and the *sRD* index according to:

$$G = 0.0254 \times sRD + 0.0037 \times T \times sRD - 0.0873 \quad \text{(Equation 1)}$$

where *G* is the instantaneous growth rate, *sRD* the standardized RNA/DNA index, and *T* the median in situ temperature below the thermocline (when present) since this depth stratum is where hake larvae are mainly found (Álvarez-Colombo et al., 2011). If thermal stratification was not detected a mean temperature for the whole water column was used. A critical *sRD* for hake larvae growth (*CsRD*) was calculated as the value corresponding to the point of inflection in which *G* goes from positive to negative values. A *CsRD* was calculated for each year using the mean temperature registered, and the percentage of larvae below this threshold was reported.
According to Buckley et al. (2008), a growth performance (*Gpf*) was also estimated as the quotient of the observed growth rate (*G*) and a reference growth rate (*Gref*) reached by a larva under optimum environmental and feeding conditions. For this we used a *Gref* calculated according to Houde and Zastrow (1993) who established a Multi-specific model based on 80 marine and estuarine species:

$Gref = 0.0106 \times T - 0.0203$ (Equation 2)

2.6. Data analysis

In the present study, variables were classified as endogenous or exogenous, and are listed in Table 2.

Because *sRD* varies along with larval development, analysis of variance with the standard length of the specimens as a covariate (ANCOVA) was performed when comparing *sRD* between sampled years (since larval size distribution was different between years).
A Two-way Analysis of variance was performed to compare the mean values of *sRD* indexes for larvae collected during both studied sampling years, taking into account their developmental stage (*DS*). *Year* and *DS* were used as factors.
Mean *sRD* and *Gpf* values obtained for larvae collected in the two surveys were calculated for Preflexion and Flexion stages of the larvae.

The relationship between the *sRD* index and the endogenous and exogenous variables was studied through Principal Components Analysis (PCA). Only 69 larvae had all the variables measured simultaneously and could be included in this analysis.

Generalized Linear Models (GLMs) were used to analyze the relationship between *sRD* index and other explanatory variables. Previously, a correlation matrix was made with all the variables (see Supplementary material 2), and the multicollinearity among them with variance inflation factors (VIF) was examined. Variables with VIFs exceeding 10 (considered as a sign of severe multicollinearity) were discarded from the modeling process (Zuur et al., 2009), as well as those strongly correlated with each other (i.e. correlations higher than 0.8). Also, the availability of potential prey was eliminated from GLM analyzes due to the low number of existing data. As a result of this process, the following variables were selected for the elaboration of GLM models: *SL*, *T*, *and L10m2* as numeric variables and *Year*, as a nominal variable. The presence of outlier data was analyzed in the modeling process and as a result, three values were eliminated from the analysis. Of

the total larvae analyzed, 360 were used in the GLM analyses. We worked under the assumption of a Gamma family error distribution and used a logarithm link function. Finally, both forward and backward stepwise regression methods were used to obtain the selected model (Faraway, 2006). The maximum likelihood method was used for choosing the final model, adopting the Akaike's lowest index model (AIC). Pseudo R2 was calculated as proof of the goodness of fit of the final model, as the quotient between the variation explained by the final model over the total variation (R2<-1-model$deviance/model$null.deviance). Deviance, used as it is linked to Kullback Leibler distance (related to variance), was only calculated for the final model obtained since the selection of models was made by Akaike's criteria. The fit of the models was evaluated in graphic form from the relationship between the linear predictor and the linearized response (See Supplementary material 3 for the selected model and Supplementary material 4 for intermediate models). All data analysis was performed using the R software (R Development CoreTeam, 2014; Libraries/packages: HighstatLib.R code, ggplot2, gridExtra, tweedie, statmod, faraway).

3. RESULTS

The survey carried out during the 2011 hake reproductive season was slightly delayed in comparison to the 2010 survey (Table 1). A wider range of larval sizes was obtained in the 2011 survey, which allowed us to describe the ontogeny of the *sRD* index of hake larvae (see RNA and DNA contents, and *sRD* plots in relation to standard length in Supplementary material 1). We observed an increase in the mean values of the *sRD* index for each developmental stage from preflexion to the transformation stage. The ANCOVA analysis using larval standard length as a co-variate showed significant differences between the mean values of the *sRD* index among the years analyzed (Table 3). In the ANOVA analysis, only the preflexion and flexion stages were compared, since larvae were not collected in more advanced stages during 2010. Significantly higher values of the *sRD* index were observed for preflexion and flexion larvae collected during 2010 (Figure 2, Table 4). Mean *Gpf* values were also higher in the 2010 survey. Mean values in 2011 were close to zero or even negative in flexion and preflexion stages, respectively (Table 5).

The critical value of the *sRD* index (*CsRD*) for the growth of hake larvae in 2010 was 1.289. Our sRD results suggest that 2% of the fish captured in 2010 were potentially starving. On the other hand, *CsRD* for 2011 was 1.305, with 35% of the larvae in starvation during this reproductive period.

The biplot representation of the PCA shows that the first component (PC1) explained 42.4% of the total variability. This component was mainly explained by larval endogenous variables related to size, weight, and growth rates (*SL*, *Age*, *W*, *RGR*, *Gr3d*), followed by exogenous variables related negatively to prey density (*NpreyEnv*, *CperStom*, *NpreyStom*) (Table 6; Figures 3A and B). Chlorophyll *a*, temperature, the biomass of *Mnemiopsis leidyi*, and density of larvae were positively related to PC1. A separation between years and *DS* (developmental stages) was distinguished along the axis of the first main component (Figures 3A and B, respectively). The second component (PC2) explained 17.6% of the total variability and was represented to a greater extent by the variability coming from the nutritional condition (*sRD* and *Gpf*) negatively, while temperature and chlorophyll *a* correlated positively and biomass of *Mnemiopsis leidyi*, and density of hake larvae were correlated negatively to this component. The third component (PC3), which accounted for 11% of the total variability, was positively related to density of larvae and availability of potential prey and negatively to nutritional condition. This opposite relationship between nutritional condition (*sRD*) and both larval and prey densities (*L10m2* and *NpreyEnv*) was evident in Figures 3A and B (lower panel). Since PC1 mainly represents the larval size, discarding PC1 allows reducing the effect of size on other endogenous variables (Ferron and Leggett, 1994). Thus, by reducing the influence of larval size, it was observed that individuals with higher *sRD* indexes were associated with lower larval densities and lower potential food densities.

After examination of multicollinearity among variables, the saturated model included the variables *SL*, *T*, *L10m2*, and *Year*. Both backward and forward stepwise procedures yielded selection of the saturated model. The backward stepwise selection procedure did not eliminate any of the variables included in the saturated model, and the intermediate models obtained through the forward stepwise selection procedure had higher AICs than the saturated model (Table 8). The graphical diagnoses of the intermediate models are shown in Supplementary material 4.

The final selected model was as follows:

$sRD \sim SL + T + L10m2 + Year$ (Equation 3)

family = Gamma(link = log)

AIC: 882.71

In the selected model of the *sRD* index, the variables *SL*, *T*, *L10m2*, and *Year* were statistically significant (Table 7, Equation 3). The standard length of the larvae was positively related to the nutritional condition. On the other hand, larval density and temperature were negatively related to the larval condition, as well as the year, indicating that larvae collected in 2010 had higher *sRD*. This model presented an R^2 of 0.55 and an AIC of 882.71. The graphical diagnosis of the link function of the model showed a good fit for the *sRD* model (Supplementary material 3).

4. DISCUSSION

The RNA/DNA ratio represents the most commonly used biochemical index as an indicator of the nutritional condition of fish larvae. The application of standardization methods has contributed to make this technique a very powerful tool (Chícharo and Chícharo, 2008). This methodology has been widely used in Clupeidae and Gadidae species, but studies on Merluccidae species are very scarce (Grote et al., 2012; Diaz et al., 2014). The present investigation on early and late larvae of *M. hubbsi* has allowed to determine the changes in the *sRD* index throughout the ontogeny of the species and to establish the relationship between the larval condition and a set of endogenous and exogenous variables.

The *sRD* index showed a positive trend from preflexion to transformation stages. The material collected in 2010 presented higher *sRD* values than those obtained during 2011, although only larvae in preflexion and flexion stages were obtained. During 2011 cruise samples were collected later in the reproductive season, which may explain the lack of older larvae in the 2010 survey. A similar pattern in the *sRD* index was observed in larval *M. paradoxus* and *M. capensis*, increasing from preflexion to

postflexion stages (Grote et al., 2012). However, there are no data of any other hake species with which to compare the evolution during the second month of life (*SL*>11 mm). The *sRD* ratio obtained during 2011 for *M. hubbsi* was somewhat lower than the values recorded by Grote et al. (2012) for larvae in preflexion, but the values obtained for larvae in flexion and postflexion stages were within the range observed for *M. paradoxus* and *M. capensis*, even higher for larvae collected during the 2010 survey.

The transition between the larval stage and the hake juvenile stage is accompanied by a change in behavior and habitat (Maynou et al., 2006). The high *sRD* observed here during the transformation stage of *M. hubbsi* could be explained by the change in the habitat (becoming demersal) and diet from larval to juvenile stages (Temperoni and Viñas, 2013; Temperoni et al., 2013). Tanaka et al. (2007) also observed a marked increase in RNA/DNA ratios towards the end of metamorphosis for *Thunnus orientalis* larvae, coinciding with a diet change.

During 2011, a significant proportion of *M. hubbsi* larvae had *sRD* values below the critical value for larval growth. In contrast, during 2010, the majority of the larvae were in good condition. When evaluating the potential explanatory variables of the *sRD* values obtained in both years, our GLM model indicated that the size and density of the larvae and temperature were significant. According to previous studies (Diaz et al., 2014), the model herein presented, showed that larval size showed a positive relationship with condition. On the other hand, temperature and larval density were negatively related to larval condition. The factor *Year* was also significant in the final selected GLM model, with higher values of *sRD* for the material collected in 2010. The PCA indicates that temperature and density of larvae are relevant variables explaining a large percentage of the variability observed, and an inverse relationship between larval condition and larval density, temperature, and abundance of potential food available in the environment was found. The inverse relationship between the nutritional condition and the availability of food is consistent with the hypothesis that zooplankton was a limiting factor in the environment. Future studies including a higher number of larvae with their associated endogenous and exogenous variables are needed to support these relationships.

Temperature stands as a relevant variable on fish larvae life traits. This variable acts directly or indirectly on biological processes and for many taxa explains a significant portion of the variability in recruitment (Houde, 2009). It controls the metabolic activity, as well as the behavior and growth of fish early stages (Blaxter, 1993). For hake larvae, a negative relationship between nutritional condition and temperature was found. While a positive relationship between larval growth rate and temperature may be expected, the relationship between *sRD* indexes and the temperature is complex and should be established for each fish species (Buckley et al., 1999). Negative relationships were also observed in other fish larvae species (Esteves et al., 2000; Do Souto et al., 2019). This phenomenon may be a consequence of compensatory mechanisms, due to the reduced activity of ribosomes at lower temperatures, and the higher content of RNA required to maintaining the same activity level (Buckley et al., 1999; Bulow, 1987).

Due to the presence of the frontal system in the study area, homogeneous coastal waters and stratified waters located towards the open sea are usually observed, with a marked thermocline during austral summer (Figure 1B). During the cruises analyzed, the average temperature of the water column in the homogeneous stations was higher (Figure 1c) than that recorded in the stratified stations (Figure 1d and e). Hake larvae are mainly found in stratified waters (Álvarez-Colombo et al., 2011). The presence of a marked thermocline in the study area is hypothesized to promote aggregations of zooplankton, ensuring high concentrations of prey for hake larvae during summer (Álvarez-Colombo et al., 2011) and offering an explanation for the inverse relationship between the larval condition and the temperature in our study.

High densities of copepod eggs and nauplii have been reported in this frontal area, as well as high densities of herbivorous calanoid copepods, which are the preferred prey for hake larvae (Viñas and Santos, 2000; Derisio et al., 2014; Temperoni et al., 2014). The herbivorous copepods likely respond to the elevated chlorophyll *a* values recorded at the thermocline depth (Viñas and Ramírez, 1996). Such densities of prey present in the stratified zone would allow greater efficiency in larval feeding since the energy costs associated with food search would be reduced. Supporting our results, Marrari et al. (2019) observed that the most favorable conditions for *M. hubbsi* recruitment and its larval survival were cold temperatures (colder than average fall temperatures) in combination with high spring chlorophyll concentrations. They suggested that the higher survival associated with colder

seasons might be the result of less competition for food between hake larvae and other plankton predators.

In the study area, ctenophore aggregations (mainly *Mnemiopsis leidyi*) seem to coincide with hake reproductive peaks (Schiariti et al., 2018). Feeding by these ctenophores may diminish the nutritional condition of hake larvae through competition for food and, ctenophores could also prey on hake eggs and larvae as well (Purcell and Arai, 2001). In this case, the ctenophores may be selectively consuming the larvae in worse condition, generating a positive bias on the observed *sRD* values. Information about these interactions is still insufficient to test these hypotheses. Biomass of *M. leidyi* was not incorporated in the GLM model here presented due to multicollinearity with other variables. However, we cannot rule out that ctenophore abundance could be a relevant driver of hake larvae nutritional condition under a different scenario.

The results presented here provide evidence of a negative relationship between the nutritional condition and larval density, which would indicate the existence of density-dependent mechanisms operating on the larval condition life traits. The average density of hake larvae in the study area in 2011 was 255±926 individuals $10m^{-2}$ (N=28 analyzed samples, mean value±SD), and 190±444 individuals $10m^{-2}$ in 2010 (N=45 analyzed samples, mean value±SD). Coincidentally, during 2011 we observed low values of the *sRD* index and the large proportion of values below *CsRD*. In addition, the maximum density obtained in 2011 (4848 individuals $10m^{-2}$) was higher than in 2010 (1992 individuals $10m^{-2}$), also representing the maximum value registered in cruises carried out in the same period between 2009 and 2017 (Ehrlich et al., 2019). Density-dependent mortality has been suggested for *Pleuronectes platessa* larvae (Hoverkamp, 1992). In *Engraulis anchoita* larvae a reduction of body condition was correlated with high larval abundances (Diaz et al., 2009). High aggregations of adult *M. merluccius*, *Phycis blennoides*, and *Micromesistius poutassou* was found to limit food resources, which was reflected in a reduction of body condition (Rueda et al., 2015). Cannibalism, which has been considered as an important factor controlling recruitment in other hake species (Jurado-Molina et al., 2006; Link et al., 2012), also cannot be ruled out as a density-dependent factor. In agreement with our results, Marrari et al. (2019) suggested that the years with higher egg

production lead to high densities of early developmental stages and consequently to higher density-dependent mechanisms reducing the survival of juveniles and later recruitment to the adult stage.

While the fitting of the final GLM model for the nutritional status of the larvae yielded results acceptable for a field study ($R^2 = 0.55$), other factors that were not considered in this work may also have influenced larval physiological state, for example the condition of the spawning females (Field et al., 2008) and the quality of the food available for the larvae (St. John et al., 2001). Further studies investigating in more detail the trophic web and the abundance of other potential predators or competitors are needed.

In conclusion, our results suggest that the nutritional status of hake larvae would be favored in cold and heavily stratified waters, and that high larval densities could increase competition for food. In years with higher larval densities, and stronger intraspecific competition, food might represent a limiting factor leading to a detriment of the nutritional condition of these larvae. To fully understand drivers of hake larvae condition, long time series studies are needed, supported by laboratory experiments under controlled conditions. The evaluation of other potentially relevant variables should be incorporated into future research.

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FOOTNOTES

[1]https://www.agroindustria.gob.ar/sitio/areas/pesca_maritima/desembarques/lectura.php?imp=1&tabla=especie_mes_2019

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

AUTHOR CONTRIBUTION

M. Diaz planned the sample collection, executed nutritional condition analysis and wrote the original the manuscript, M. Do Souto performed the statistical analysis, P. Betti performed larval age and growth determinations and participated during the samplings, B. Temperoni performed zooplankton quantifications and larval gut contents, A. Schiariti determined jellyfish biomass, L. Machinandiarena determined larval abundances, D. Brown supervised larval age and growth determinations and revised the manuscript. G. Macchi contributed to planning and critical revisions of the manuscript.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest and that the research meets the required ethical guidelines.

TABLES

Table 1. Collection data for the ichthyoplankton samples from 2010 and 2011 research surveys used in this study. N: number of larvae analyzed, T: mean temperature below the thermocline (when present) in centigrade degrees, Depth: maximal depth at the sampling station, MEB: Epibenthic Sampler, RMT: Rectangular Midwater Trawl. See details in the text.

Date	Net	Hour	Lat. (°S)	Long. (°W)	Depth (m)	N	T (°C)
01/26/10	Bongo	15:35	44.42	64.97	77	7	12.76
01/27/10	Bongo	11:05	44.14	64.59	70	21	12.28
01/27/10	Bongo	14:27	43.81	64.27	68	21	11.83
01/27/10	Bongo	20:53	43.49	64.37	55	13	13.61
02/01/10	MEB	17:45	43.74	63.50	70	34	10.28
02/02/11	Bongo/RMT	21:40	44.46	64.95	76	29	14.60
02/07/11	Bongo	6:25	43.04	62.56	75	2	13.25
02/07/11	RMT	23:47	44.13	63.81	75	27	13.95
02/08/11	Bongo	6:15	44.11	64.23	55	1	14.96
02/08/11	Bongo	11:20	43.82	65.02	73	4	14.51
02/08/11	MEB/RMT	12:50	44.00	64.41	70	34	14.70
02/08/11	Bongo	16:30	44.15	64.58	67	11	14.90
02/08/11	Bongo	19:50	43.84	64.28	46	14	16.08
02/09/11	Bongo	0:45	43.55	64.66	56	6	15.37
02/09/11	Bongo	2:27	43.33	64.23	69	25	14.50
02/09/11	Bongo	5:28	43.47	63.92	69	7	13.95
02/09/11	MEB	11:39	43.75	63.55	84	16	13.34
02/09/11	RMT	21:45	43.99	63.12	68	53	14.27
02/10/11	MEB	12:18	43.54	63.28	76	70	14.55

Table 2. Variables employed in this study were classified as endogenous or exogenous. Abbreviations, units, and number (N) of data points.

Endogenous variables	Acronym	Unit	N
Standard length	***SL***	mm	395
Larval age	***Age***	days	145
Larval dried weight	***W***	µg	395
The otolith recent growth rate: average of the width of the increments of the last 3 days	***RGR***	µm	145
The growth rate of the last 3 days	***Gr3d***	mm day^{-1}	145
Trophic incidence (number of preys per stomach)	***NpreyStom***		166
Carbon milligrams per stomach	***CperStom***	mgC	166
Standardized RNA/DNA	***sRD***		363
Growth performance	***Gpf***		363

Exogenous variables	Abbreviation	Unit	N
Mean temperature (below the thermocline)	***T***	°C	395
Surface chlorophyll *a*	***Chla***	mg m^{-3}	395
Potential prey availability (copepods and its developmental stages smaller than 2 mm)	***NpreyEnv***	individuals m^{-3}	197
Biomass of the potential predator ctenophore *Mnemiopsis leidyi*	***Nem***	ml *M. leidyi* m^{-3}	395
Hake larvae density	***L10m2***	individuals $10m^{-2}$	395

Table 3. Analysis of variance of mean values of the standardized RNA/DNA index (*sRD*) for *Merluccius hubbsi* larvae collected during the reproductive peak surveys carried out in 2010 and 2011. Standard length (*SL*) of the specimens was used as co-variate (ANCOVA).

sRD	SS	df	MS	*F*	*p*-value	Coef.
Model	462.80	2	231.40	202.38	<0.0001	
Year	369.61	1	369.61	323.26	<0.0001	
SL (mm)	239.33	1	239.33	209.32	<0.0001	0.25
Error	411.62	360	1.14			
Total	874.42	362				

Table 4. Two-way Analysis of variance of the mean values of the standardized RNA/DNA index (*sRD*) for *Merluccius hubbsi* larvae collected during the reproductive peak surveys carried out in 2010 and 2011. *Year* and developmental stage (*DS*) were used as factors.

sRD	SS	df	MS	*F*	*p*-value
Model	356.29	2	178.14	161.46	<0.0001
Year	355.22	1	355.22	321.94	<0.0001
DS	26.27	1	26.27	23.81	<0.0001
Error	289.08	262	1.10		
Total	645.37	264			

Table 5. Mean values (± Standard error) of the standardized RNA/DNA index (*sRD*) and growth performance (*Gpf*) obtained for *Merluccius hubbsi* preflexion and flexion larvae collected during the reproductive peak surveys carried out in 2010 and 2011. Number of larvae analyzed between parentheses.

Variable	2010		2011	
	Preflexion	Flexion	Preflexion	Flexion
sRD	3.49±0.15 (67)	4.18±0.19 (16)	0.86±0.13 (84)	1.52±0.08 (98)
Gpf	1.46±0.14 (67)	2.03±0.14 (16)	-0.26±0.04 (84)	0.12±0.06 (98)

Table 6. Principal Component Analysis (PCA) performed using the endogenous and exogenous variables recorded for hake larvae collected in 2010 and 2011: standard length of specimens (*SL*), age of the specimens (*Age*), dry weight (*W*), RNA/DNA index (*sRD*), growth performance (*Gpf*), average temperature below the thermocline (*T*), recent otolith growth rate (*RGR*), growth rate of the last 3 days (*Gr3d*), concentration of chlorophyll *a* (*Chla*), milligrams of carbon per stomach (*CperStom*), trophic incidence (*NpreyStom*), availability of potential prey (*PreyEnv*), biomass of *Mnemiopsis leidyi* (*Nem*), density of hake larvae (*L10m2*). Eigenvectors and correlation of the variables with the main components (PC1, PC2 and PC3) and their eigenvalues (av) are indicated.

Variable	PC1 Correlation	(av=5.94) Eigenvector	PC2 Correlation	(av=2.46) Eigenvector	PC3 Correlation	(av= 1.54) Eigenvector
SL	-0.97	-0.40	0.06	0.04	-0.06	-0.05
Age	-0.91	-0.37	0.10	0.06	-0.07	-0.06
W	-0.87	-0.36	-0.01	-0.01	-0.13	-0.11
sRD	-0.22	-0.09	-0.83	-0.53	-0.46	-0.37
Gpf	-0.22	-0.09	-0.82	-0.53	-0.48	-0.39
T	0.43	0.18	0.44	0.28	-0.74	-0.59
RGR	-0.85	-0.35	-0.06	-0.04	0.04	0.03

Gr3d	-0.71	-0.29	-0.10	-0.06	0.12	0.10
Chla	0.39	0.16	0.58	0.37	-0.52	-0.42
CperStom	-0.71	-0.29	0.24	0.15	-0.25	-0.20
NpreyStom	-0.70	-0.29	0.17	0.11	-0.19	-0.15
NpreyEnv	-0.73	-0.30	0.32	0.20	0.30	0.24
Nem	0.39	0.16	-0.45	-0.29	0.11	0.09
L10m2	0.20	0.08	-0.36	-0.23	0.22	0.18

Table 7. Generalized Linear Model of the nutritional condition (*sRD*) of *Merluccius hubbsi* larvae collected during 2010 and 2011. Levels of significance: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' '.

	Estimate	Std. Error	t value	Pr(>|t|)	
Intercept	1.783e+00	2.735e-01	6.519	2.45e-10	***
SL	1.195e-01	7.461e-03	16.022	< 2e-16	***
T	-8.961e-02	2.019e-02	-4.438	1.21e-05	***
L10m2	-2.622e-05	6.434e-06	-4.075	5.69e-05	***
Year 2011	-1.313e+00	7.544e-02	-17.405	< 2e-16	***

Table 8. Generalized Linear Model of nutritional condition (*sRD*) of *Merluccius hubbsi* larvae collected during 2010 and 2011. Akaike's information criterion (AIC) obtained for each step through forward stepwise regression method for model selection.

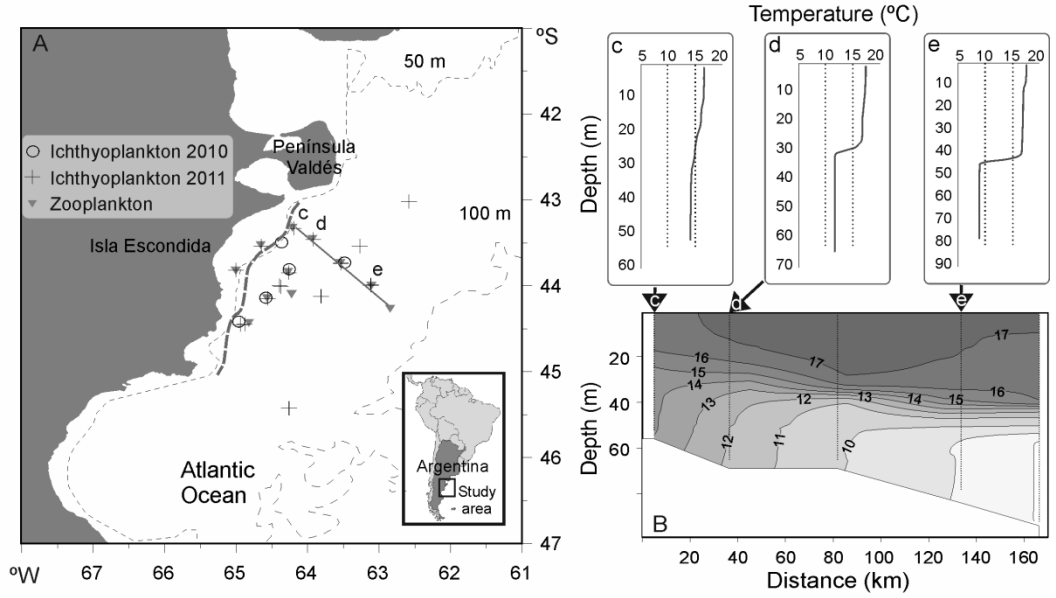
Step	Model	AIC
Start	*sRD* ~ 1	1173.44
1	*sRD* ~ *Year*	1092.23
2	*sRD* ~ *Year* + *SL*	900.42
3	*sRD* ~ *Year* + *SL* + *T*	895.87
4	*sRD* ~ *Year* + *SL* + *T* + *L10m2*	882.71

FIGURE LEGENDS

Figure 1. (A) Location of zooplankton and ichthyoplankton sampling stations where *Merluccius hubbsi* larvae were collected in two surveys carried out in 2010 and 2011 during the reproductive peak of hake in January. Gray dashed line shows the typical position of the Península Valdés tidal front (critical Simpson parameter $\phi = 40$ J m^{-3}, Simpson, 1981). (B) Typical vertical section of temperature (°C) in the study area during January (black solid line in Figure 1a; black arrow indicates position of vertical profiles in Figures 1c,d,e), with a homogeneous coastal zone and a stratified zone at greater depths. (c) Vertical temperature (mean values; °C) profile in the homogeneous coastal zone (see position in Figure 1A). (d) and (e) Vertical temperature (mean values; °C) profiles in the most external stratified zone (see positions in Figure 1A).

Figure 2. BoxPlot of the standardized RNA/DNA index (*sRD*) according to the developmental stages of *Merluccius hubbsi* larvae collected during the reproductive peak surveys carried out in 2010 and 2011.

Figure 3. Biplot graph of the first three main principal components (PC1, PC2 and PC3) obtained from the Principal Component Analysis (PCA) performed using the endogenous and exogenous variables recorded for hake larvae collected in 2010 and 2011: standard length of the specimens (*SL*), age of the specimens (*Age*), dry weight (*W*), RNA/DNA index (*sRD*), growth performance (*Gpf*), average temperature below the thermocline (*T*), recent otolith growth rate (*RGR*) , growth rate of the last 3 days (*Gr3d*), concentration of chlorophyll *a* (*Chla*), milligrams of carbon per stomach (*CperStom*), trophic incidence (*NpreySto*), availability of potential prey (*PreyEnv*), biomass of *Mnemiopsis leidyi* (*Nem*), density of hake larvae (*L10m2*). (A) The *Year* in which the samples were collected is distinguished. (B) Larval developmental stage is distinguished.



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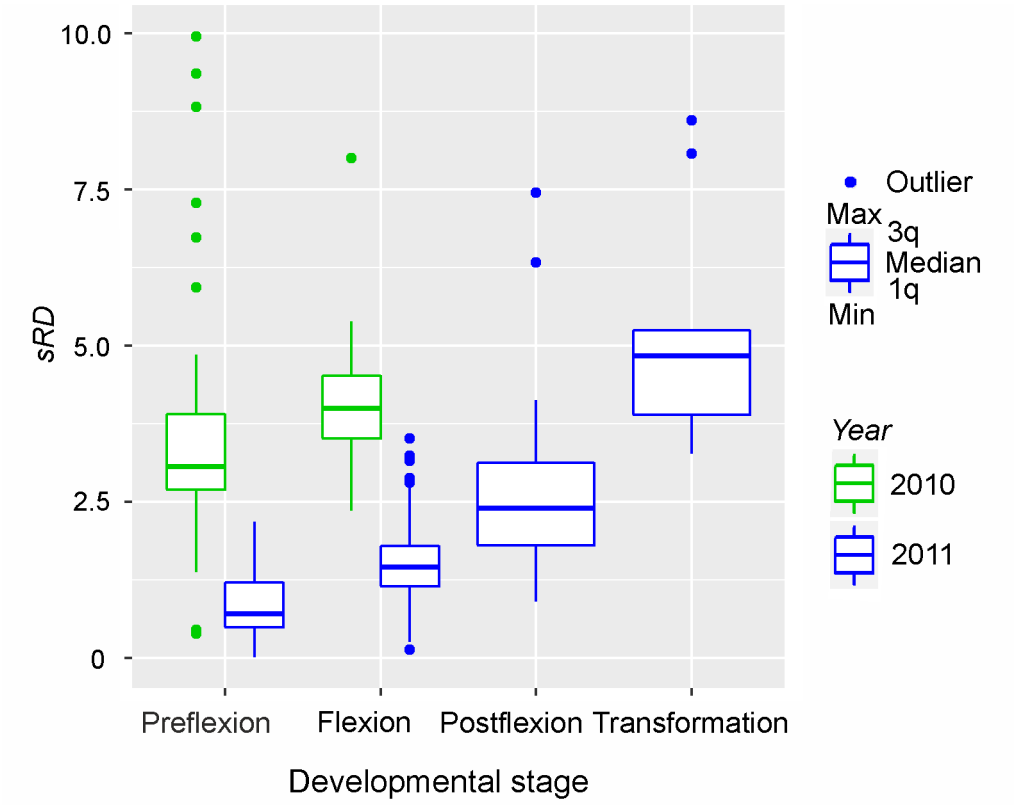

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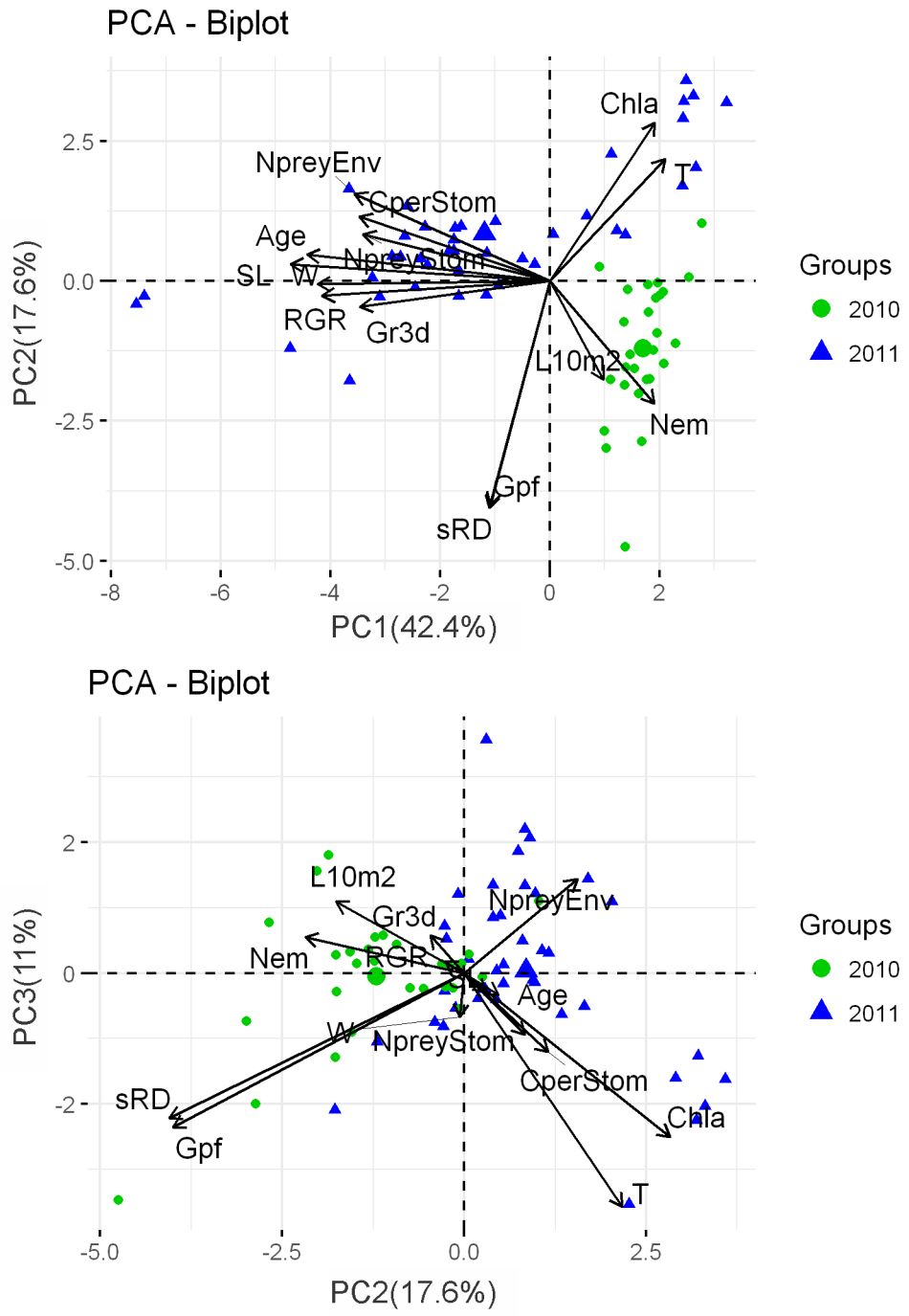

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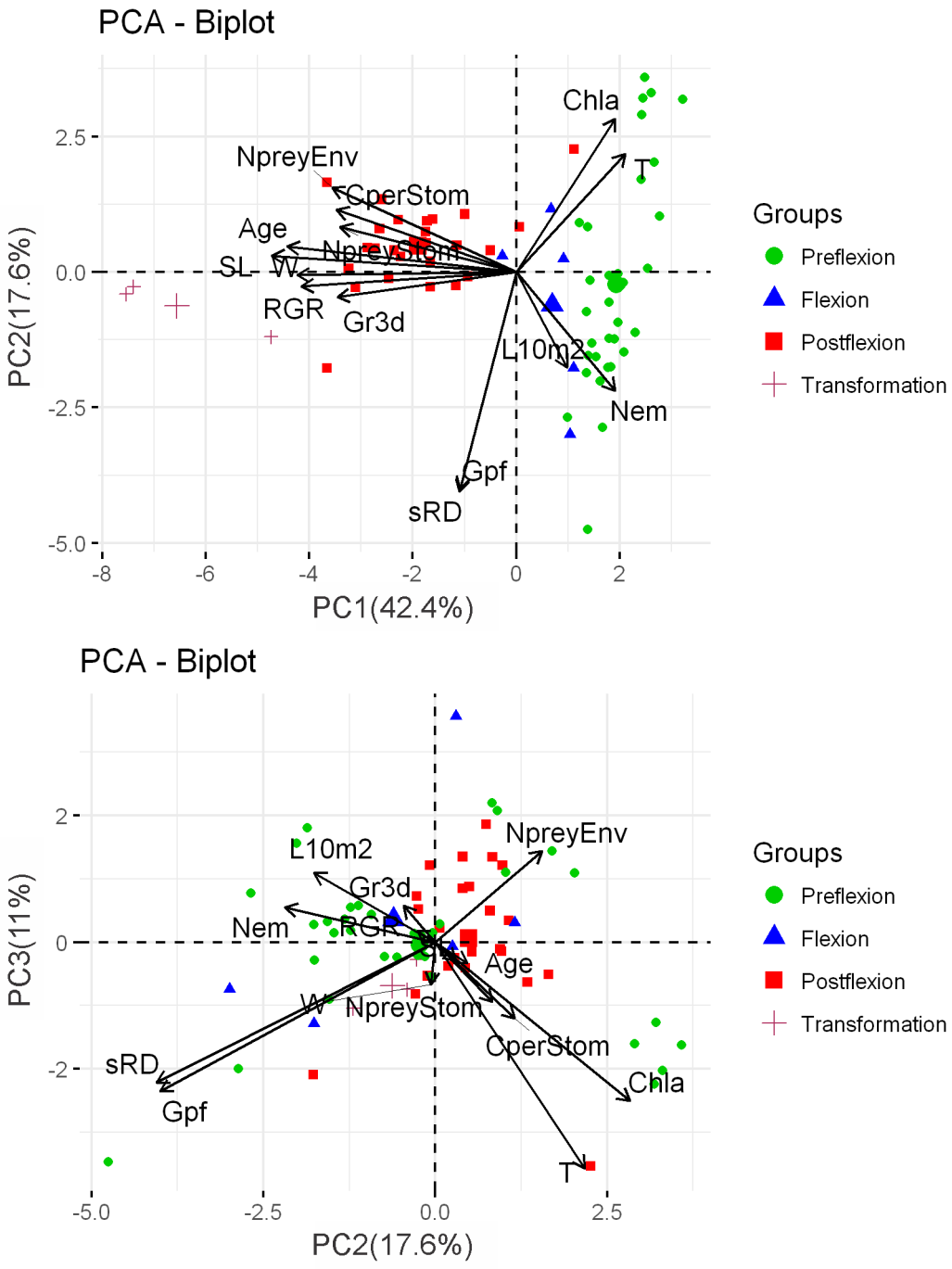

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