 To prevent us from sounding preachy, I think the order of material should be:

1. Intro

2. Case study, with general commentary about how to interpret the model ecologically (but not BP yet)

3. Simulated date

4. PLS best practices - drawing on the \_results\_ of the case study and simulation, e.g., you can see overfitting

5. Conclusions and future directions

# Introduction

Data collected on many variables for the same individuals presents some unique challenges for data analysis. Multivariate data is not new to the field of ecology, and ecologists have been using techniques like principal component analysis to reduce dimensionality and simplify the interpretation of multivariate data for a long time (CITATION TO SOME OLD PCA PAPER). However, in recent years, the scale of data available for ecological research has increased due to advancements in high-throughput sampling technology (Kallenbach et al. 2014; Kfoury et al. 2017), image processing (Berger et al. 2010; Fahlgren et al. 2015), automated and remote data logging (Cooke et al. 2004; Porter et al. 2005), remote sensing (Roughgarden et al. 1991; Aplin 2005), high-throughput sequencing technologies (Soininen et al. 2009), and citizen science (Silvertown 2009; Bonney et al. 2009; Dickinson et al. 2012). Simultaneously, there is perhaps and increasing interest in going beyond simply describing multivariate data to inferring processes from patterns.

One typical approach to multivariate statistical inference in ecology is to first reduce dimensionality through an unsupervised technique like PCA, and then to look for visual separation in a score plot or perhaps to use the derived latent variables (principle components in the case of PCA) as predictors in a statistical test for a relationship with some response variable. However, this may not answer the question the researcher is actually asking. Unsupervised techniques like PCA are agnostic to response variables and can only describe the variation in data. PCA followed by a statistical test on principle components answers the slightly different question “What are the main axes of variation in the data? Do those axes have a relationship with the response variable?” This approach can be justified when one is truly interested in the axis that describes variation. For example, the leaf economics spectrum (LES) is a highly repeatable principle component that explains variation in leaf traits from a slow to fast rate of return on investments (Wright et al. 2004). Because this multivariate trade-off exists across all plants and within groups of plants, it is reasonable to ask questions about how position along the LES varies among habitats, along environmental gradients, or among clades. However, a failure to find a relationship with the LES does not mean that measured leaf traits do not vary among habitats, along environmental gradients, or among clades.

Supervised approaches, on the other hand, take response variables into account and test how response variables co-vary with one or more predictor variables. Using a supervised technique answers a fundamentally different question than the unsupervised approach described above. When using a supervised analysis, you are answering the question “Is there a multivariate relationship between the predictors and the response variable?” This may seem a minor distinction, but can lead to completely different conclusions, and can even miss statistically significant relationships, as we will demonstrate.

The use of supervised multivariate analyses in ecology may not be as common because of some of the challenges ecological data typically creates, including missing values, multicollinearity, small sample sizes, and more variables than observations (AKA the “curse of dimensionality”). Previously, these challenges limited the use of supervised multivariate statistics, but this is no longer the case. In fact, several techniques, including partial least squares regression (PLSR) and its discriminant analysis extension (PLS-DA), handle the above-mentioned challenges of ecological data especially well. Unlike PCA, which creates axes that explain the most variation in the data, PLS creates axes that explain the most co-variationwith a dependent variable. This is an important distinction because it is not safe to assume that the best explanatory variables will also show the most overall variation among samples.

PLSR was first developed in the late seventies in the field of econometrics (Wold 1975). and later adopted by analytical chemistry (Geladi and Kowalski 1986), but has only been widely adopted in ecology by the sub-field of chemical ecology (Hervé et al. 2018). The underlying assumption of PLS is that covariation between the predictor variable(s) and the response variable(s) is due to a small number of “latent” variables. For this reason, the technique has been readily adopted for the analysis of metabolomic data because a change in a large number of metabolites may be a result of a change in a single enzyme or metabolic pathway. In fact, PLS has been implemented into many metabolomics-specific statistical software (de Souza et al. 2017). However, the utility of PLSR is not limited to metabolomic data, or even to data all of one type. In fact, one of the strengths of PLSR is that, unlike permutational MANOVA for example, it does not rely on distance or dissimilarity measures and therefore retains information about the relative importance of individual variables which can be used to make loading plots and summarized with a variable importance in projection (VIP) score. VIP scores have been shown to be very robust to determining which predictor variables are responsible for variation in the response variable (Chong and Jun 2005). This makes PLS an ideal technique for highly multivariate ecological datasets where multicollinearity may be present, and the researcher would not only like to address a hypothesis about the effects of some independent variable on a multivariate dataset, but also to determine which measured variables are most responsible for the multivariate relationship.

To demonstrate the use of PLSR and compare with a PCA approach, we employ a case study as well as simulated data…

# Case Study

Muir et al. (2017) collected data on leaf traits of 16 *Solanum* species grown from seed. Measured traits included leaf mass per area (LMA), leaf thickness, leaf dry matter content (LDMC), stomatal conductance (*gs*), mesophyll conductance (*gm*), assimilation rate (*A*N), water use efficiency (WUE), maximum rate of carboxylation (*V*cmax) and leaf respiration (*R*dark). In their analysis, Muir et al. performed PCA to recapitulate the leaf economics spectrum—a highly repeatable principle component axis of leaf traits describing a tradeoff in metabolic rates and investment to leaf structure (Wright et al. 2004). They then used the first principle component (the LES) to ask questions about how the position along the LES varies among habitats and phylogenetic relationships. Instead, we will use the data to answer a different question—do leaf traits vary among habitats, namely with mean annual precipitation and temperature?

## Methods

*Solanum* leaf trait data and coordinates of species habitats are available on Dryad (citation). We used the habitat coordinates to download mean annual precipitation and temperature data from WorldClim (Fick and Hijmans 2017). Muir et al. (2017) identified one species, *S.* *juglandifolium*, as a potential outlier and performed their analyses both with and without that species. For the sake of brevity for this case study, we only performed analyses with this species excluded.

To replicate the approach of the original paper, we performed PCA on leaf traits to recapitulate the LES. Then, the first principle component axis (i.e., the LES) was used as a predictor variable in regressions with habitat temperature or precipitation.

PLS regression was used to test the hypothesis that there is a relationship between climate variables and leaf traits. Although it is possible to include multiple response variables in PLS models to account for collinearity, we chose to perform PLS regressions for temperature and precipitation separately to improve interpretability. PCA and PLS regression were performed using the *opls* function in the *ropls* package in R with default settings other than increasing the number of permutations to 1000 for PLS (Thévenot et al. 2015).

For PCA, a component is retained if the variance explained is greater than the mean variance explained by 10 components. For each PLS component an R2Y value is calculated to describe the explanatory power of the component. Additionally, through 7-fold cross-validation, a Q2 value is obtained for each component, which can be interpreted as the predictive power of the model (Eriksson et al. 2006). Components are retained until either the R2Y (the amount of variation in the response variable explained by the component) is less than 0.01 or the Q2 value is less than 0.05. A p-value is calculated for both cumulative R2Y and Q2 values by permutation.

## Results and Discussion

The PCA of leaf traits results in two principle components with a cumulative R2 of 0.558. PC1, which explains 32% of the variation in the data, effectively recapitulates the LES with a strong positive correlation with LMA, LDMC, and leaf thickness and a strong negative correlation with A, gm, Vcmax and gs. [one sentence explanation of what this means]. This principle component varies significantly with habitat temperature (F(1,63) = 9.07, p = 0.004), but not with precipitation (F(1,63) = 1.22, p = 0.273).

PLSR produced a single component model for both precipitation and temperature. Unlike the PCA regression analysis, both precipitation and temperature produced significant models (table). These components explain a lot less variation in the data (R2X) compared to the PCA (R2 = 0.558). This is because PLS is instead maximizing the R2Y, the amount of variation in precipitation or temperature explained by the leaf traits. For both temperature and precipitation, R2Y is low, but significantly higher than chance (table). Additionally, Q2 values for both models are similar to R2Y values, indicating an absence of overfitting. Adding more variables will always increase R2Y, but not necessarily increase Q2 (citation).

Rdark loads weakly onto PC1 but is negatively correlated with temperature in the PLS model.

# Simulated data

## Methods

To demonstrate some of the properties of PLS and PCA, we used randomly generated multivariate data created with different covariance structures. All multivariate datasets had 20 observations, one factor with two levels (10 observations per level), and 25 continuous variables. All 25 variables had a variance of 1 and a mean of 0 when they were not discriminating between factor levels. Covariance and the difference in means between factor levels was adjusted depending on the scenario:

1 ) “Null”: 5 variables with covariance of 0, and two groups of 10 variables with a covariance of 0.5 (Fig 1A).

2) “Needle in a haystack”: two groups of 10 variables with covariance of 0.5 and 5 variables with a difference in means of 2 (Fig 1D).

3) “Control” where two sets of 5 variables covary moderately with covariance = 0.5 and discriminate between groups (difference in means = 2); 5 variables with covariance = 0.5 and no difference in means; and 10 variables that do not covary or distinguish groups (i.e. noise) (Fig 1G).

Multivariate data were simulated in R using the *holodeck* package (citation), which allows simple generation of multivariate data frames with varying correlation structures. We created 100 randomly generated datasets using the same parameters under each of these scenarios. PCA and PLS-DA were conducted using the *opls* function from the *ropls* package with default settings (Thévenot et al. 2015). PCA regression was performed by using all significant principal components as predictors in a linear model with the factor as the response. For PCA regression and PLS-DA, root mean squared error of prediction (RMSEP) was calculated by 7-fold cross-validation. For PLS-DA, p-values were calculated by permutation testing using 500 permutations. In the case that a PLS or PCA model couldn’t be created for a dataset (for example, because the first component was not significant), those datasets were removed after recording the number of failed models.

To test accuracy of identification of important discriminating variables, we used the “control” and “needle in a haystack” scenarios where variables were either discriminating or not. We set criteria for both methods (PCA and PLS-DA) to identify important discriminating variables. For PLS-DA, a variable was considered identified as discriminating if it had a variable importance in projection (VIP) score greater than 1 (Chong and Jun 2005). For PCA, a variable was considered discriminating if its distance from 0 in a correlation plot of the first two principal components was greater than 0.38, which is equivalent to the threshold Pearson correlation coefficient that would be significant at alpha = 0.05. We then compared these to known variable identities (discriminating or not) and created a confusion matrix for each dataset with the number of discriminating variables correctly identified as important being a true positive. From this we calculated a kappa coefficient for each dataset, which describes the accuracy of the method for choosing discriminating variable. A Kappa coefficient of 1 indicates complete accuracy while a kappa of 0 indicates important variables are selected no better than by chance. A negative kappa indicates that selection of important variables is worse than chance. See supplemental files for reproducible R scripts.

## Results and Discussion

*PLS-DA is prone to overfitting.* PLS models should be cross-validated to determine model predictive power and statistical significance. In the *ropls* package this is done through 7-fold cross validation where some of the data is left out for model generation, then predicted by the model and compared to true values. Cross validation is used to select a number of components. In the case that zero components are selected as optimum, the model should be considered non-significant. If at least one component is appropriate, an R2X, R2Y, and Q2 value will be calculated and p-values generated by permutation testing. R2X is the variation in the predictor variables explained by the model (analogous to R2 for PCA), R2Y is the variation in the response variable explained by the model (analogous to R2 for a regression), and Q2 describes the predictive power of the model and is generated through cross-validation (Eriksson et al. 2006). A Q2 close to 1 means the model has strong predictive power and a Q2 value close to or below zero means the model has weak predictive power. If Q2 is much lower than R2Y or close to or below zero, this is an indication that the model has very low predictive power and there is probably not a significant relationship between the response and predictors (Thévenot et al. 2015). A p-value is also reported by the *ropls* package by randomly permuting data labels and re-calculating R2Y and Q2 and measuring the proportion of permuted models with R2Y and Q2 values higher than the model created with the true data.

Under the null scenario, both PCA and PLS-DA are expected to find no separation between groups. Due to a lack of convergence, 6 of the PCA regression models failed. The remaining 94 models explained a mean For PLS-DA, 75 out of 100 models failed because the first predictive component was not significant (Thévenot et al. 2015). The PLS-DA models that were successfully built have low R2y and Q2 values, which is an indicator of poor model performance (table). Despite this, the permutation tests of Q2 are marginally significant on average (pQ2 = 0.080 ± 0.054). In the case that a model has poor predictive power (low Q2) it is recommended that no p-value should be reported, because permuted Q2 values are more likely to be higher when the true Q2 is very low, resulting in more frequent false positives. Additionally, a score plot should not be shown when models are non-significant or have low predictive power because even highly non-significant PLS-DA models will show some separation in score plots (Fig1C).

A close up of a map

Description automatically generated

Figure 1: Multivariate analysis of representative datasets from three data scenarios: “null” (A, B, C), “control” (D, E, F) and “needle in a haystack” (G, H, I). The first column shows correlation heatmaps (A, D, G). Variable names on the axes that begin with “C” were generated with a covariance of 0. 5, those that begin with “N” were generated with a covariance of 0, and those that begin with “D” were generated to have different means in the two groups. The second column shows PCA score plots (B, E, H). The third column shows PLS-DA plots (C, F, I). For PLS-DA plots, the first two predictive axes are plotted, Q2 values are calculated using 7-fold cross validation, and pQ2 is calculated with 500 permutations (C, F, I). Ellipses represent 95% confidence bounds, parenthetical numbers on axis labels are the percent of total variation explained by the axis. Note that in C, the PLS-DA is clearly not a good model due to low Q2 value. We recommend not including a such PLS-DA score plot for non-significant results in a publication.

*PLS-DA finds obvious differences between groups just as well as PCA*. Under the control scenario, the PCA for two of the datasets failed due to a convergence error and PLS-DA models were built for all 100 datasets. Both PCA and PLS-DA show significant separation between groups (table). In a PCA score plot, there is clear visible separation between groups along PC1 (Fig. 1D). For PLS-DA, both mean R2Y and mean Q2 are high (0.835 ± 0.061 and 0.752 ± 0.072, respectively), and permutation testing is highly significant (pQ2 = 0.002 ± 0.001). Because the discriminating variables are also the variables that contribute the most to overall covariation in the dataset, PCA and PLS-DA are nearly equivalent, despite answering slightly different questions. Here, PCA is answering the question “Is there a main axis of variation in the data?” and then we are able to evaluate separation along that axis visually through a score plot or statistically by doing a t-test on principal component axis scores. PLS-DA, on the other hand, is answering the question “What variables (if any) explain the difference between groups?” and it answers this directly.

*PLS-DA outperforms PCA when discriminating variables aren’t responsible for the majority of covariation.* For the needle in a haystack scenario, there is poor separation between groups in the PCA score plot (Fig 1H) while PLS-DA shows strong separation (Fig 1I) and has a very high mean R2Y and Q2 value (0.906 ± 0.046 and 0.709 ± 0.108, respectively) and a highly significant permutation test (p = 0.002 ± 0.001).

In the needle in a haystack scenario, PCA performs poorly at finding group separation, while PLS-DA is able to find strong separation between groups. This is because the variables that contribute to differences between the groups are not contributing greatly to the overall variation in the data. Again, this is by design because these methods answer different questions. PCA is finding the main axis of variation in the data, while PLS-DA is finding variables that co-vary with group membership. This is further demonstrated through calculation of the kappa coefficients for these analyses.

*PLS-DA identifies discriminating variables while PCA identifies variables with high variation.* Kappa coefficients for the two methods (Fig 2. table?) show that PLS identifies variables that were created with different means in the two levels of our factor consistently better than PCA. Even in the control scenario where PCA and PLS performed similarly in finding separation between groups, PLS far outperforms PCA at correctly identifying the variables most responsible for that separation (Fig 2A). This is by design since PCA is agnostic to the response variable and just attempting to explain variation in the data while PLS-DA is specifically identifying which variables (if any) contribute to differences between the groups.

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Figure 2. Distribution of Cohen’s Kappa for the control (A) and needle in a haystack (B) scenarios. A value of 1 means perfect identification of discriminating variables by the model while a value of zero indicates the model performed no better than random chance.

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