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Exploring subindividual variability: role of ontogeny, abiotic environment, and seed-dispersing birds.

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Abstract

- Within-individual trait variation -otherwise known as subindividual variation- is an important component of phenotypic variation, with a genetic and epigenetic basis. We explore its adaptive value and the effects of ontogeny and the environment on subindividual variability.
- We conducted a field study to analyze the effects of tree age, soil pH, soil water content, and soil nutrients on subindividual variability in fruit size of Hawthorn (*Crataegus monogyna*) in three sites in Northwestern Spain. Additionally, we examined how bird-mediated selection influences average and subindividual variation in fruit size.
- Results show that average and subindividual variation of fruit size were related to fitness affecting seed dispersal. Older trees produced larger fruits, but tree age did

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not affect subindividual variation in fruit size. Abiotic environmental factors differently affected subindividual variation and average fruit size. Seed-dispersing birds exerted correlated selection on fruit size average and variation, favoring trees with larger and less variable fruit size at one site.

- Our work suggests that the fruit size variation within individual trees, the subindividual variation, is modified by abiotic environmental factors and additionally it is an adaptive trait that responds to natural selection.

Keywords: *Crataegus monogyna*; Correlated selection; Functional trait; Phenotypic plasticity; Selection coefficient; Selection gradient; Subindividual variability; Subindividual variation; Soil nutrients; Soil water content; Tree age.

Introduction

Plants produce multiple copies of the same organs such as leaves, flowers and fruits, which are re-runs of the same individual genotype under different micro-environmental conditions (Castellanos et al., 2008; Herrera 2009). These copies are not phenotypically identical, and subindividual variation can be substantial (Larrinaga and Guitian, 2016; DeSoto et al. 2017; Herrera 2017).

Subindividual variability is mainly due to organ level phenotypic plasticity (Winn 1996; Herrera and Bazaga 2013) and developmental noise (Primack 1987; Peters et al. 1988; Diggle 1995). Environmental gradients within an individual, such as light or temperature disparity among different parts of the plant, are a cause of organ-level phenotypic variation (Niinemets, 2003; Murray, 2005; Mal and Lovett-Doust 2005). However, it is unknown whether differences among individuals in ontogeny or environment can affect subindividual variation.

Subindividual variation is known to have a genetic (Herrera et al., 2015; Kulbalba et al., 2017) and epigenetic basis (Herrera and Bazaga, 2013; Alonso et al., 2017). Additionally, subindividual variation can affect fitness (Herrera et al., 2015; Arceo-Gómez et al., 2017), for example, by enhancing whole-plant photosynthetic performance and optimizing the exploitation of resources (Givnish, 1988; Hollinger, 1996; Osada et al., 2014). Therefore, it has evolutionary implications (Simons, 2009; Sobral et al., 2013; Herrera et al., 2014). Furthermore, subindividual variation changes the spatial structure of functional plant traits (Herrera, 2017) and can be considered a functional trait itself sensu (Violle et al., 2007). Thus, in the same manner that variation among individuals broadens the ecological breadth of species and enhances community-wide functional diversity (Sides et al., 2014), subindividual variation is also consequential for the functional ecology of plant populations and communities (Herrera, 2017).

Plant trait subindividual variation may be expected to be selected by interacting animals, because animals would tend to prefer lower levels of variation in reward, for example the variation of energy intake when ingesting fruit (Herrera 2009). This tendency leads to a positive selection for subindividual variation from antagonistic interactions, such as herbivory, and a negative selection for subindividual variation from mutualistic interactions, such as pollination or seed dispersal (Sobral et al., 2010). The total selection for subindividual variation of a given trait would also depend on the composition of the animal guild (Sobral et al., 2013). For example, a diverse mutualistic guild with diverse preferences would eventually lead to a positive selection for subindividual variation (Herrera 2009). However, the existence of correlated selection (the selection of associations between traits as described by Lande and Arnold, 1983) has never been investigated for average and subindividual variation.

We studied fruit size subindividual variation of hawthorn (*Crataegus monogyna*). We examined how current abiotic environmental factors at the tree level (namely soil water content, soil nutrients, and soil pH) affected the subindividual variation and average fruit size, while studying the selective pressures exerted by seed-dispersing birds at three sites in NW Spain (S1 Fig). We hypothesize first that abiotic environment will affect average and subindividual variation of fruit size differently. Because fruit size and variation might also be influenced by resource allocation limitations (Leishman, 2001.; Violle et al., 2009), we controlled for ontogeny (tree age) and crop size (the number of fruit or seeds per individual) in our models.

We additionally hypothesize that there is correlated selection on the average and variation of fruit size exerted by seed-dispersing birds, because previous work showed at least independent selection for both traits (Sobral et al., 2010 and 2013). Specifically, we expected that trees with larger average fruit size and smaller variation would be selected for. However, among trees with small average fruit size, birds would select those with higher variation. These effects would create a selection landscape on the combination of average and variation of fruit size.

To our knowledge, we find for the first time that average and variation of a trait, at the within-individual level, are affected by different environmental factors while having fitness consequences. These findings substantiate the adaptive value of subindividual variation as a functional trait with potential eco-evolutionary implications.

Materials and Methods

Hawthorn (*Crataegus monogyna*, Rosaceae) is a shrub or small tree with fleshy fruit containing a single seed. It is distributed over most of Europe, northern Africa, and western Asia (Lang, 1987). We selected three sites in Northwestern Spain in 2006: Hospital (42°42'N, 7°06' W, 1260 m asl), Mostad (42°37'N 7°09'W, 859 m asl), and Carucedo (42°30' N, 6°49' W, 550 m asl). At each site, 30 hawthorn trees were haphazardly chosen (S1 Fig; at the Carucedo site, the same trees were chosen as in Sobral et al., 2010).

Soil chemical properties

Soil was sampled under each of the 90 trees selected with a soil borer in spring 2006 (to avoid the pulse of nutrients following leaf shedding). To account for the possible small-scale heterogeneity of soil properties, we extracted three samples per tree (270 samples) from the layer 15- 30 cm deep. We performed pH and nutrient analysis from the composed samples (one per tree, i.e. 90 samples). We analyzed the pH, potential acidity (pH_{KCl}), assimilable phosphorous quantity, several cations (Ca^{2+} , Mg^{2+} , Na^+ y K^+), and carbon and nitrogen quantity. Carbon and nitrogen were only measured for one third of the samples due to logistic limitations. Samples were air dried and sieved through a 2-mm mesh net. The pH and pH_{KCl} were measured with a pHmeter Crison micropH 2001 in a soil-water mixture in a 1:2.5 ratio after 10 minutes for pH and after two hours for pH_{KCl} (Guitian and Carballas, 1976). Cations were extracted using a NH_4Cl 1M solution (Peech, 1947), and their concentrations were determined in an atomic absorption spectrophotometer (Perkin - Elmer 2380). Total carbon and nitrogen percentages were determined with a Leco 1000 CNS autoanalyzer. Finally, assimilable phosphorous was extracted using a NaHCO_3 0.5 M solution (Olsen, 1982). Its concentration was measured using the phosphomolybdic blue complex colorimetry in a Vitatron MCP machine (wavelength 880 nm).

Soil moisture

Soil water content was sampled on two occasions per tree: in spring and autumn of 2006. On each occasion, we took a 100 g sample from the superficial layer (15-30 cm depth) and a 100 g sample from the deeper layer B (30-50 cm depth) at three different points under each tree (6 samples per tree; 1,680 soil samples in total). Samples were weighed fresh, then dried in a drying oven for 24 hours at 105°C and reweighed. We estimated soil water content as (fresh weight- dried weight)/ dried weight.

Tree age

We estimated tree age using standard dendrocronology protocols (Stokes, 1996). We extracted a 5 mm diameter wood core from each tree using a Pressler drill, dried and polished the samples, and then counted the number of growth rings using a magnifying glass. Cores were taken at breast height (1.3 m) to have comparable ages within the study and all the selected trees had a single stem. The Hawthorn wood proved very hard and samples were extremely difficult to obtain and easy to break; only 54 out of the 90 age measurements were usable (11, 19, and 24 trees from Carucedo, Hospital, and Mostad respectively). The measurements of the 54 trees retained for analysis are presented in S1 Table. Hawthorn trees in the studied sites are not large, so we preferred to take only a wood core from each tree so as not to damage them.

Phenotypic traits

Birds swallow fruits in a longitudinal fashion; thus, the size constraints imposed by gape width are determined by fruit diameter rather than length (Wheelwright 1993). Additionally, diameter and length of fruits are under different phenotypic selective pressures: fruit diameter is the target of bird selection, while fruit length is indirectly selected due to correlations between traits (Sobral et al., 2010). For this reason, this study focuses on fruit diameter, hereafter referred to as fruit size.

Before the beginning of the dispersal season (October 2006), we haphazardly collected 50 fruits per tree. The diameter of each fruit was measured with a 0.01 mm precision caliper. The fruits were kept refrigerated and measured within one week after collection. A total of 4,500 fruits, belonging to the 90 trees selected, were measured.

We quantified the subindividual variation of fruit size using the coefficient of variation (CV; the individual standard deviation divided by its average and multiplied by 100), because variance and standard deviation are scale-dependent and therefore cannot be used to compare variation levels (Pearson, 1901).

Selection exerted by seed-dispersing birds

In Europe, hawthorn fruit are mostly consumed by blackbird (*Turdus merula*), redwing (*Turdus iliacus*) and song thrush (*Turdus philomelos*, Lang 1987). They consume fruits smaller than 13 mm in diameter (González-Varo and Traveset, 2016), which was always the case in our sampled trees and populations (see Table S1).

Between October and December 2006, we identified the seed-dispersing birds at our sites by conducting 369 total hours of bird observations (123 hours of observation per site and an average of 4 hours of observation per focal tree). Number of fruits consumed in our focal trees and bird species were recorded.

To assess phenotypic selection by seed-dispersing birds, we used the success of seed dispersal per tree as a fitness component. In long-lived organisms, it is a common approach to estimate fitness from a measure of reproductive success (Kingsolver et al., 2001), such as dispersal success (Sobral et al., 2010 and 2013). The rationale being that trees with a higher number of dispersed seeds pass more genes to subsequent generations since dispersed seeds are more likely deposited in suitable microsites and escape negative density dependent effects, such as competition and predation (Howe and Smallwood, 1982). Furthermore, passing through dispersers' digestive tracks has been found to improve germination (Traveset et al., 2008). During the 369 observations in our focal trees and additional observations in this system (Sobral et al., 2010) we have never observed secondary seed dispersal by mammals feeding on

fallen fruits, (mice do predate on seeds but they do leave the exocarps in situ so it is possible to count them). We have neither observed hawthorn seeds within mammal feces in the study sites.

To estimate the seed dispersal success per tree, we quantified the number of fruits, or number of seeds given that both are equal in this species, (hereafter 'crop size') and the area under the canopy before fruit consumption by animals started. In each tree, we haphazardly marked five branches and counted their fruits. We marked three 0.5×0.5 m quadrats under each tree to estimate the number of fallen fruits weekly for the duration of the experiment. In December, after the dispersal event, only rotten fruits were left on the branches, the rest of which were dispersed or had fallen. After the dispersal event, we counted the number of fruits remaining on the marked branches (the number of fruits, or predated exocarps, in the quadrats under the canopy were counted weekly).

Statistical Analyses

We quantified the within and between tree and site components of fruit size via analysis of the components of variance. For that fruit diameter was analyzed in a model in which tree identity nested within site and site were included as fixed factors.

We analyzed the effects of tree age and abiotic factors (soil water content, soil pH, and soil nutrients) on plant phenotypes (average fruit size and subindividual variation of fruit size). To reduce the number of variables and avoid collinearity effects, we performed a principal component analysis (PCA) on the set of edaphic nutrients (P^- , Mg^{+2} , Ca^{+2} , Na^+ , K^+). Carbon and nitrogen were excluded since we could only measure these nutrients in a third of the samples due to logistic restrictions – however carbon and nitrogen were highly correlated to the other nutrients. We extracted the first principal component from the set of nutrients (P^- , Mg^{+2} , Ca^{+2} , Na^+ , K^+) with eigenvalues higher than 1, which explained 63% of the cumulative variance of the sample; this component is called *soil nutrients*. Since soil pH and potential acidity were highly correlated (Spearman $r > 0.9$), we retained soil pH for further analysis. Soil water content per tree was assessed as the average soil water content in autumn and spring samples (i.e. three samples per tree and period). The results did not change when analyzing soil water content in spring and autumn independently.

Phenotypic plant traits (average and subindividual variation of fruit diameter) were the response variables in two General Linear Models using type III sum of squares and the restricted maximum likelihood method (the residuals fitted the normal distribution). Predictor variables were crop size, soil water content, soil nutrients, soil pH, and tree age. Crop size was included to account for the potential effects of the correlation between fruit number and size or variation. Additionally, site and its interaction with soil properties were included as factors in the models. The interaction of tree age with site was also included and later discarded because effects were not significant, and the model fit was worse when including that interaction. Model selection was performed using the AICc criterion.

Phenotypic selection analyses

Phenotypic selective pressures were assessed following classic methods (Lande and Arnold, 1983), as has been done before in this system (Sobral et al., 2010, 2013 and 2014). The relative fitness of tree t was defined as the proportion of dispersed seeds of tree t relative to the mean number of dispersed seeds per tree at each site. We estimated the number of dispersed seeds per tree as the initial crop size of a tree minus the number of fruits fallen to the ground and the fruits remaining in the canopy after the dispersal event. Number of fallen fruits was estimated as the average fallen fruit density across the quadrats under the canopy during the study period (counted weekly) for the projected area of the canopy. Number of fruits remaining on the tree was assessed using the initial and final count of fruit on the five branches surveyed per tree and extrapolating that difference to the initial crop size.

Thus, at each site, we analyzed the relationship between the relative fitness (relative dispersal success) of a tree with both the average fruit size and the fruit size sub-individual variation.

We analyzed the total and correlated selection gradients respectively S and γ_{ij} (Lande and Arnold 1983), on average fruit size and on fruit size subindividual variation. Total linear selection differentials were assessed as the standardized coefficients of the simple regression of each trait (average or subindividual variation) on the relative fitness (dispersal success) of each tree per site. Correlated selection gradients were assessed as the slope of the interaction of both traits on the relative fitness of each tree per site. These gradients were assessed by means of a multiple regression that, besides the interaction between traits, included the simple effects of the traits (Lande and Arnold 1983). These gradients are later variance standardized (multiplied by the standard deviation of the traits) to be comparable among traits. This analysis was independent of the analyses considering tree age and abiotic environment, so we could include a larger number of trees (15 trees were discarded because of fruit fungal infections; the final number of trees used in the selection analyses was 75). Selection differentials and gradients were analyzed separately for each site.

Results

During the 369 hours of field observation, we recorded 943 consumption events (*Crataegus monogyna* fruit consumed by birds) in our focal trees. The species observed consuming hawthorn fruit were the blackbird (35.4 % of the fruit consumed), the redwing (57.9 %), and the song thrush (6.7 %).

The analysis of the components of variance showed that 49.8 % of the variance in fruit diameter was due to among site differences, whereas 22.3 % was due to among tree differences, and 27.9% was due to within individual differences. See distributions of average fruit size and subindividual variation of fruits size per population in Fig S2.

Older trees produced larger fruits, but subindividual variation of fruit size was not related to tree age. Soil nutrients had a marginally significant positive effect on average fruit size whereas soil pH had a negative effect. Trees with larger crops had lower levels of subindividual variation. Fruit size variation generally decreased with soil pH and the effects of water content depended on site (Table 1, Fig 1).

Models explained 71% of the variance of average fruit size and 60% of the variance of subindividual variation. Site explained a larger part of the variance for average fruit size than for subindividual variation (43% and 22% of the variance of both variables respectively). Tree age, crop size and abiotic environment explained the additional 28% of average fruit size and 39% of the variance of subindividual variation.

We found significant correlated selection (selection gradient γ_{ij} at Hospital, Table 2) and marginally significant total linear selection (selection differentials S in Table 2), which in general were positive for average fruit size and negative for subindividual variation, although this varied among sites. Birds dispersed more seeds from trees with larger and less variable fruit size, causing correlated selection on average and subindividual variation –which favored trees with larger and homogeneous fruits at Hospital (Table 2, Fig 2). As we expected, among trees with small average fruit size, birds selected those with higher variation. Thus, we found a selection landscape in which subindividual variation is beneficial to dispersal when average fruit size is small but decreases dispersal success when average fruit size is large (see Fig 2.). At the other sites, we found marginally significant evidence for either positive selection on average size (Carucedo, Table 2) or negative selection on subindividual variation (Mostad and Hospital, Table 2).

Discussion

We show for the first time that seed-dispersing birds exerted correlated selection on the average and variation of fruit size, favoring trees with large and homogeneous fruits at one of the study sites (Table 2). We found correlated selection only in Hospital, likely because the among individual distribution of average and variation in this population allowed an optimal combination of both to be selected. That was not the case in other populations (See Fig. S2). The potential effect of different selective forces at the sub-individual level across different nearby populations may be diluted by gene flow. Sobral et al. (2010, 2013 and 2014) already found total selection on subindividual variation, but the present study is the first to analyze the effect of ontogeny and current environment on subindividual variation as well as to find correlated selection on fruit average size and variation. Hereafter we discuss the ecological and evolutionary implications of our findings.

Negative selection on subindividual variation is the expected outcome of a mutualistic relationship because variability in reward (in this case, the energy intake from consumed fruit) affects animal perception (Herrera 2009). Yet for diverse animal assemblages, intra-plant

variability may increase the possibilities of interaction with multiple animal species. Selective pressures exerted by seed-dispersing birds have been found to explain part of the differences between subindividual fruit size variation among hawthorn populations across the European latitudinal range (Sobral et al., 2013).

The frugivore assemblage studied here has a poor functional and phylogenetic diversity because all species belong to the *Turdus* genus and are roughly the same size. Importantly, these species have a similar gape width, which is a key trait in bird-fruit interactions as legitimate seed dispersers swallow the entire fruit. Therefore, we would expect different results in other systems where frugivore assemblages are more diverse, particularly in terms of gape width. For example, an increase in intra-individual variability could be advantageous under such scenarios, because many different dispersing species, even species with narrower gapes, could feed on the fruits of an individual tree (Escribano-Ávila et al., 2013).

To control for potential biases due to causal relationships between seed number and size or variation due to sampling effect or resource allocation (Leishman, 2001; Violle et al., 2009) we included crop size (i.e. the number of fruits/seeds) and tree age in the models. Average fruit size, but not its variation, increased with tree age. The positive effect of plant age on reproductive traits has been documented, for example, for crop size (Wender et al., 2004) and flower production (Herrera 1991, Ehlers and Olesen 2004). Effects can reach a limit due to senescence. Although it might not be possible to extrapolate tree age outside of our study (see methods for details), trees in this study had probably not reached senescence. Our age measures ranged from 9 to 54 years old, and the expected life span for *Crataegus monogyna* in Europe is 97 years (Schweingruber and Poschlod, 2005).

Soil water content affected subindividual variation in a different manner between populations, which differed in their soil water content (see table S1). In Carucedo more water content meant less subindividual variation in fruit size, whereas in Mostad the opposite effects occurred and in Hospital there was not any effect. The result in Carucedo may reflect that reducing soil water availability causes different levels of desiccation in fruits—water is unevenly disseminated in modular plants and, as a result, higher subindividual variation in fruit size. In Mostad, soil water availability was higher than in Carucedo, and more water was related to higher subindividual variability in fruit size. This may be because after overcoming the water limitation level, processes of competition for resources might occur among fruits. Past studies have shown that the effect of soil moisture on fruit size, or related traits, is very variable. Soil water content increased fruit size in olives (Michelakis, 1989) and increased flower and seed production in *Lavandula latifolia* (Herrera 1991) but not in *Ipomopsis aggregata* and *Linum lewissii* (Burkle and Irwin, 2009).

We found a marginally positive effect of soil nutrients on average fruit size and no effect on its variation. This could be because we analyzed the nutrients altogether and different nutrients could have different effects on fruit size and related traits. For example, potassium had a positive effect on fruit size in *Malus domestica* (Nava and Dechen, 2009) and in *Citrus volkamerina* (Quaggio et al., 2002) while nitrogen had a negative effect on fruit size, and phosphorous presented a non-linear effect on fruit size in *Citrus volkamerina* (Quaggio et al., 2002).

We found that a more basic soil pH lead to a decrease in fruit size and in fruit size variation. This could be the result of within-individual environmental filtering (Violle et al., 2012), i.e. a reduction of phenotypic variance due to harsh conditions. Alkalinity has been found to have a negative influence on fruit production and fruit size in blueberries (Austin and Bondari, 1992).

Our works shows that average and variation of fruit size within an individual hawthorn are differently affected by abiotic and ontogenetic factors and have independent and correlated effects on fitness. Subindividual variation originates from micro-environmental variation within a single genotype (Herrera, 2009). Thus, our results imply that the potential plasticity of a genotype is a trait itself with its own ecological and evolutionary causes and consequences. Subindividual variation is an important component of biodiversity with eco-evolutionary consequences yet to be understood.

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Table 1. Final model results analyzing the factors that affect average fruit size and subindividual fruit size variation (n = 54 trees). *Soil nutrients* is the principal factor extracted from the PCA in the set of nutrients measured (see methods). Crop size is the number of fruits/seeds. B values show the sign and size of the effect, they can be compared between them since they were assessed on standardized variables. Model selection was performed using the AICc criterion. Significant (p < 0.05) or marginally significant (p < 0.10) selection coefficients are shown in bold and italics respectively.

	Explained variance	B	se	Wald Chi-square	df	p value	
Average fruit size (mm)	71%	Site		41.616	2	0.000	
		Tree age	0.191	0.0823	5.408	1	0.020
		<i>Soil nutrients</i>	0.266	0.1433	3.453	1	<i>0.063</i>
		Soil pH	-0.305	0.1501	4.136	1	0.042
Subindividual fruit size variaton (CV)	60.4%	Site		1.587	2	0.000	
		Crop size	-0.953	0.204	2.176	1	0.000
		Soil pH	-0.234	0.531	5.234	1	0.022
		Soil water content	2.460	0.927	2.653	1	0.103
		<i>Site * soil pH</i>			5.430	2	<i>0.066</i>
		Site* soil water content			1.566	2	0.000

Table 2. Total linear selection differentials (S) and correlational selection gradients (γ_{ij}) on average fruit size and fruit size subindividual variation in each of the study sites. Selection coefficients were variance standardized (multiplied by the trait standard deviation). Significant (p < 0.05) or marginally significant (p < 0.10) selection coefficients are shown in bold and italics respectively. A 10% confidence level was chosen due to the limited sample size per site (17, 29, and 29 trees per site respectively).

		Standardized coefficient	se	pvalue
Carucedo	<i>Total linear selection differential on average fruit size (S)</i>	0.868	0.767	<i>0.078</i>
	Total linear selection differential on subindividual variation (S)	0.105	0.234	0.844
	Correlated selection gradient (γ)	3.723	0.367	0.585
Hospital	Total linear selection differential on average fruit size (S)	0.024	0.252	0.891
	<i>Total linear selection differential on subindividual variation (S)</i>	-0.315	0.109	<i>0.057</i>
	Correlated selection gradient	-7.378	0.184	0.001
Mostad	Total linear selection differential on average fruit size (S)	-0.087	0.303	0.625
	<i>Total linear selection differential on subindividual variation (S)</i>	-0.319	0.083	<i>0.059</i>
	Correlated selection gradient (γ)	-1.303	0.116	0.473

Figure 1. Relationship between average fruit size (upper panel) and subindividual variation of fruit size (lower panels) with the ontogenic (tree age), correlated traits (crop size; i.e. number of seeds), or environmental characteristics (soil nutrients, soil water content, and pH) that affected them. Circles refer to Carucedo, triangles to Hospital, and squares to Mostad. Only significant or marginally significant effects are represented following data on Table 1.

Figure 2. Phenotypic selection landscape showing the correlated selection (γ_{ij}) exerted by seed-dispersing birds on average fruit size and their variation at the Hospital site (n = 29 trees).

S1 Fig. Map of the study sites in NW Spain.

S2 Fig. Distributions of subindividual variation of fruit size and average fruit size per tree in each of the study sites. Histograms are drawn from the 54 trees retained for the analyses on environmental and ontogenetic effects.

S1 Table. Environmental characteristics per tree, tree age, and tree phenotype. This table shows the results of the 54 trees (out of 90) retained for analyses since they included data for all covariates.

