

1 Experimental designs for testing the interactive effects of
2 temperature and light in ecology and the problem of periodicity

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Abstract

The abstract must not exceed 350 words and should list the main results and conclusions, using FOUR simple, factual, numbered statements: 1: set the context for and purpose of the work; 2: indicate the approach and methods; 3: outline the main results; 4: identify the conclusions and the wider implications.

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Introduction

Across the tree of life, temperature and light availability shape a number of important biological processes including growth and metabolic rates (MacLean & Gilchrist, 2019) sex determination (Brown *et al.*, 2014), acclimatization to seasonal environments (Hamilton *et al.*, 2016) and the timing of life cycle transitions (phenology) (Forrest & Miller-Rushing, 2010). These biological responses in turn dictate broad scale ecological processes and patterns ranging from biogeochemical cycling (Piao *et al.*, 2007) to species range limits (Chaine & Beaubien, 2001). Characterizing the specific dynamics of how these environmental factors synergistically affect biological processes across a wide range of taxa has become even more important as anthropogenic global change continues to expose organisms to novel environmental conditions (Pörtner & Farrell, 2008).

Because temperature and light availability often co-vary in the field (for example, in most temperate ecosystems, daylength and temperature both increase as the season progresses (Rosenberg, 1974)) it can be difficult to disentangle their relative contributions to biological processes. In contrast, experimental manipulations of climate variables in artificial environments can mechanistically characterize biological responses to environmental fluctuations (Ettinger *et al.*, 2020; Primack *et al.*, 2015). Growth chambers of all shapes and sizes have been used to this end (Downs, 1980) and these efforts have greatly advanced researchers' understanding of the fundamental biology of a wide variety of organisms and their ability to predict the responses to current and future climate change (Stewart *et al.*, 2013).

However, controlled environment experiments have their own challenges. Experimentalists must balance biological realism with statistical inference, experimental effort with statistical power, and account for the effects of unmanipulated or unmeasured variables (Scheiner & Gurevitch, 2001). Because biological responses to the environment are generally the product of complex interactions between multiple environmental signals (Casal, 2002), seemingly small choices about experimental design can generate significant differences in outcomes. Experimental treatments are rarely standardized among researchers even within disciplines (Wolkovich *et al.*, in Review.) and these complexities may in part contribute to many discrepancies between experimental studies and ob-

servation data (Poorter *et al.*, 2016). Even with these limitations, growth chamber studies remain a powerful tool for mechanistically assessing organismic responses to the environment provided that the implications of treatment designs are well understood and well matched with the scope of the research question.

As technology advances and experiments become more complex, researchers can manipulate more variables and multiple axes of variation (e.g. temperature, amplitude, periodicity, wavelength) at the same time. Yet these efforts may tradeoff between biological realism and statistical inference. Through investigating the literature of experiments with plant phenology (timing of recurring life cycle events, e.g. leaf budburst, flowering), we found that experiments that manipulate both photo- and thermo- periodicity often introduce a latent covariation between light and temperature experimental treatments that may misrepresent the effects of each of these environmental variables, and the interaction between them. Below, we begin by briefly detailing how temperature and light treatments are generally applied in experimental phenology studies and review the minimum experimental elements required to robustly test interactions between two or more environmental variables. We then detail the problem of inference that can arise manipulating the periodicity of both temperature and light in experiments and demonstrate the extent to which this is an issue with mathematical and experimental examples. Finally, we conclude by outlining several possible solutions for overcoming these issues. While, our example deals with phenology of temperate woody plants, the issues and solutions we present below are broadly applicable to studies of any other organisms and biological processes that utilize temperature and daylength signals.

Estimating phenological cues from experiments

Decades of experimental work in growth chambers have demonstrated that temperature (both cool temperatures in fall/winter and warming temperatures in spring) and photoperiod are the primary cues of phenology for plants in the temperate/boreal zones (Ettinger *et al.*, 2020). While exposure to cool winter temperatures (chilling) strongly impacts phenology (Laube *et al.*, 2014), we focus here on warm temperature treatments and light treatments, because controlled chilling treatments with light are uncommon ((Wolkovich *et al.*, in Review.)). Choices that about how to apply these two treatments in particular that can compromise inference on their effects, so we will focus of these two cues.

While a large variety of experimental designs have been used to study plant phenology, generally phenology experiments tend to manipulate two major axes of light and warm temperature variation:

1. Intensity: The amount or quality of a variable. Here we define temperature intensity as the amount of heat present in the system (measured in degrees). In the phenology literature this measurement is generally referred to as forcing. We define light intensity as the luminosity or irradiance present in the system (measured in lumens or watts).
2. Periodicity: The interval at which the intensity of the variable is applied. Hereafter, we refer to the periodicity of light as photoperiod (often used synonymously with “daylength”) and

the periodicity of temperature as thermoperiod.

For phenology, photoperiodicity is generally considered the primary light cue for plants ((Way & Montgomery, 2015), though see (Brelsford & Robson, 2018; Cober *et al.*, 1996) regarding light intensity and phenology). For temperature, conventionally both intensity and periodicity drive phenological activity and several metrics (e.g. growing degree hours, thermal sums, growing degree days) that combine these two axes have been developed (Gu, 2016). The importance of thermo-intensity and periodicity is well supported; under natural conditions diurnal temperature fluctuations in temperate regions can be quite large in the spring, and studies have found that diurnal temperature variation strongly influences plant phenology (Burghardt *et al.*, 2016). In fact, even if thermoperiodicity is not an explicit treatment variable (i.e. manipulated systematically), incorporating it in experiments is essential for translating experimental results into real world predictions (Chiang *et al.*, 2020).

Like many other biological processes, recent advances have demonstrated that plant phenological responses are nonlinear, due largely to interactions between cues (Wolkovich *et al.*, in Review.; Fu *et al.*, 2015), highlighting the need for experiments designed to evaluate the strength of these interactions. To have the statistical power to partition the individual and interactive effects of two or more variables, an experiment must:

1. Have at minimum of two treatment levels of at least two variables.
2. Treatment levels must be full factorial (Fig. 1a.). Full factorial designs are both balanced (Fig. 1b.) and orthogonal (Fig. 1c.); which is to say that all possible treatment combinations are applied and each treatment is independent of all others (Cheng, 2016).

These two critical elements may seem obvious but can be conspicuously absent from many published studies. In the case of woody plant phenology, using a recently published database (OSPREE: Observed spring phenological responses in experimental environments (Wolkovich *et al.*, 2019)) we found that out of 152 controlled environment experiments (across 93 studies) only 18 manipulated both light and forcing cues with a design that was both balanced and orthogonal. This notable dearth of robust tests of light and temperature interactions may stem from the common limitations of time, space, and resources that experimentalists often face, but it may equally relate to a fundamental issues that arises from the fact that these variables themselves are comprised of multiple axes of variability.

The problem of periodicity

A common approach in phenology experiments that seems to balance prior knowledge about the underlying physiology of phenology, biological realism and experimental inference is to vary photoperiodicity, and thermal intensity and periodicity (e.g. Flynn & Wolkovich, 2018; Sanz-Perez *et al.*, 2009; Basler & Körner, 2014). For example, a basic experiment might include a long (12 hours)

and short (8 hours) photoperiod treatment and a high (30/20°C day/night) and low (20/10 °C day/night) temperature treatment. Note that in this case, the thermoperiodicity is not an explicit treatment (both high and low temperature treatments employ a diurnal fluctuation of 10 °C), and is simply incorporated in the design to enhance biological realism. At first glance, this design appears to meet the criteria of a full factorial design, multiple treatment levels that are balanced and orthogonal, with mean high/low temperature treatments (25 and 15 °C respectively) and long/short photoperiod treatments applied in all possible combinations.

Yet the orthogonality of this design is based on the assumption to a 12 hour thermoperiod. If, rather the thermoperiod is coupled with the photoperiod, this is not the case because the daily mean temperature of the long/high treatment will be higher than that of the short/high treatment, and the long/low treatment slightly warmer than the short/low. This is because the warmer day time temperatures are applied for different duration across the high temperature treatments. While this covariation among the photoperiod and temperature treatments is biologically realistic, it makes it statistically impossible to differentiate their independent and interactive effects on any given biological process.

This problem of inference that arises from the experimental covariation of thermo- and photoperiodicity is not limited only to studies seeking to directly compare the effects of photoperiod and forcing; it applies in any study evaluating the influence of photoperiod on biological activity, even if it is the only manipulated cue. Experimentally isolating the effect of photoperiod assumes that all other environmental variables are held constant. Similar to the case described above, the covariation of photoperiod and thermoperiod in an experiment where forcing was supposed to be a consistent ambient condition, would yield a situation in which longer photoperiod treatments were also receiving more, unmeasured heating than the shorter photoperiod treatments. In this case, some amount of the perceived photoperiod effect is due to the latent, increased forcing, and the true effect of photoperiod cannot be ascertained.

Of the 51 experiments in the OSPREE database that manipulated photoperiod experimentally, up to 43% of them appear to include a covariation with thermoperiod. Of the 18 studies that manipulated both photoperiod and temperature interactively, we found that up to 55% of them may have this issue, suggesting that the true interactive effects of these cues on spring phenology is still quite poorly characterized. This may be in part why the relative contribution of temperature and photoperiod cues to spring phenology remains a contentious debate in the phenology literature (Koerner & Basler, 2010).

Periodicity and inference

If the lack of orthogonality introduced to experiments when photoperiod and thermoperiod covary is overlooked, regression models will overestimate the photoperiod effect and underestimate the forcing effect on spring phenology (Fig. 2a,b.). If experiments are designed to quantify the interaction between photoperiod and forcing, here too, the interactive effect will be underestimated

due when compared to the true effect (Fig. 2c,d.). This is, in part because forcing is the variable with latent variation, and because studies repeatedly suggest that forcing is a more dominant cue than photoperiod for spring phenology (CHUINE *et al.*, 2010; Zohner *et al.*, 2016; Gauzere *et al.*, 2019).

We can mathematically solve for how much of an estimated photoperiod effect is due to forcing—in experiments where they covary—by making several major assumptions. If we assume forcing and photoperiod effects are additive, linear and there is no interaction between the two effects, then we can solve (algebraically) for the estimated effect of forcing and photoperiod given an orthogonal treatment design (see Fig. 2, Supporting info). Using estimates from one experiment that covaried forcing and photoperiod effects Flynn & Wolkovich (2018), we found roughly two-thirds of the estimated photoperiod cue could be due to forcing effects.

While we are aware of no experiments that explicitly compare the effects of co-varying vs. independent photo- and thermo- periods, two phenology experiments in our lab utilized many overlapping treatment levels and species from the same sampling sites however in one study, Flynn & Wolkovich (2018), photo- and thermo- period co-vary, while in the other Buonaiuto & Wolkovich (2021) photo- and thermo- period were varied independently. Comparing the cue estimates from these two studies offers an opportunity to test the theoretical and mathematical predictions, and further understanding the uncertainty in cue estimate due to periodicity.

We subset each dataset to include only the species shared among the two studies, and re-analyzed the data using Bayesian hierarchical models to compare difference in the photoperiod and forcing estimates (see Supplement for Methods). We found that the estimated differences in the mean response to photoperiod and forcing among study designs were on the same order as our mathematical predictions, and that the un-coupled design estimated a weaker (less negative) photoperiod effect, and stronger forcing effect than the coupled experimental design (3,??).

There are almost certainly other factors driving the differences between these experiments. Both were conducted in different years, sampled different individuals from the population, and used different methods for applying chilling pre-treatments (Flynn & Wolkovich, 2018; Buonaiuto & Wolkovich, 2021). However, because this comparison is well matched to our mathematical predictions and prior knowledge about how temperature and photoperiod are expected to interacting in phenology, we argue that the influence of periodicity covariation on statistical inference is apparent enough to take seriously.

Paths Forward

Above we have systematically demonstrated that experiments that co-vary thermoperiod and photoperiod cannot robustly differentiate the individual effect of temperature and photoperiod on a

spring phenology (or any other biological process) or accurately quantify their interactive influence. Given the paucity of interactive studies in the literature, it is clear that more well designed studies will be needed to better characterize the effects of these cues. Below we offer several generalized experiment designs that improve statistical orthogonality of controlled environment experiments which could be further developed and adjusted to fit the needs of experimentalists across many sub-fields of biology.

1. **Covarying photo- thermo- period with quantified uncertainty.** It may be that the experimental design that best balances environmental realism, statistical inferences and translatability to observational studies are designs that co-vary periodicity to mimic natural systems (4a.). Moving forward, researchers using this design need to be aware of the non-orthogonality of this design, and be sure to present the uncertainty surrounding their cue effect estimates, which could be done using a similar mathematical approach to the one we present in this paper (see Supplement).
2. **Manipulate photoperiod and temperature intensity with no thermoperiodicity.** This approach allows for the maintenance of statistical orthogonality across treatment combinations (4b.). The main drawback is that this design sacrifices the biological realism of diurnal temperature variation, which may make it more difficult to translate estimates from experiments to real world applications.
3. **Compensatory diurnal temperature fluctuations.** There are almost unlimited pairs of integers that can reduce to the same mean (e.g. $(24 + 26)/2 = (30 + 20)/2 = 25$) and the non-orthogonality of the mean daily temperature that arises in a coupled photo-thermoperiod design could be corrected for by proportionately increasing the diurnal temperature fluctuation of the short photoperiod treatment relative to the long treatments (4c.). However, if the differences between day and night temperature has a meaningful biological effect, this introduces another confounding, non-orthogonal factor for interpreting temperature and photoperiod effects. For example, the influence of day time warming of phenology can be as much as three times stronger than proportionate night time warming (Rossi & Isabel, 2017; Meng *et al.*, 2020).
4. **Uncouple thermoperiod and photoperiod.** By varying thermoperiod and photoperiod independently (4d.), statistical orthogonality can be maintained across treatment. However, this approach may also introduce new artifacts that occur from the biological rather than statistical interactions between light and temperature (Chew *et al.*, 2012). For example, there is evidence that increasing temperatures in the first two hours of daylight can be almost as effective for stimulating shoot elongation as similar temperature increases for the whole photoperiod (Erwin, 1998). With this design, treatments must inherently differ in the amount of time the warmer daytime temperature extends into the dark nighttime light regime, introducing a new axes of non-orthogonality.

In correcting one problem, each of these designs introduces another, which may in fact be an intrinsic property of any experimental manipulation. It would certainly be useful for researchers to explicitly

test how cue estimates vary among these experimental designs, and which design is most useful for predicting phenology in the field under current and future climate conditions. In the meantime, we hope this treatment of the issue reminds experimentalists that we must continue to be thoughtful about matching design to the goals of a study, and being transparent about uncertainty around our experimental inference.

Author contributions

DMB, MD and EMW conceived of the manuscript; MD and EMW developed the algebraic solution; DMB performed the comparative analysis of the published studies; DMB led the writing of the manuscript. All authors contributed to writing and gave final approval for the submission.

Data Availability

Data from the Flynn & Wolkovich (2018) study is available at the Harvard Forest Data Archeive (<https://harvardforest1.fas.harvard.edu/exist/apps/datasets/showData.html?id=HF314>) and from the Buonaiuto & Wolkovich (2021) study available at Knowledge Network for Biocomplexity (<https://knb.ecoinform>). The R code used to analyse the data is available on github.

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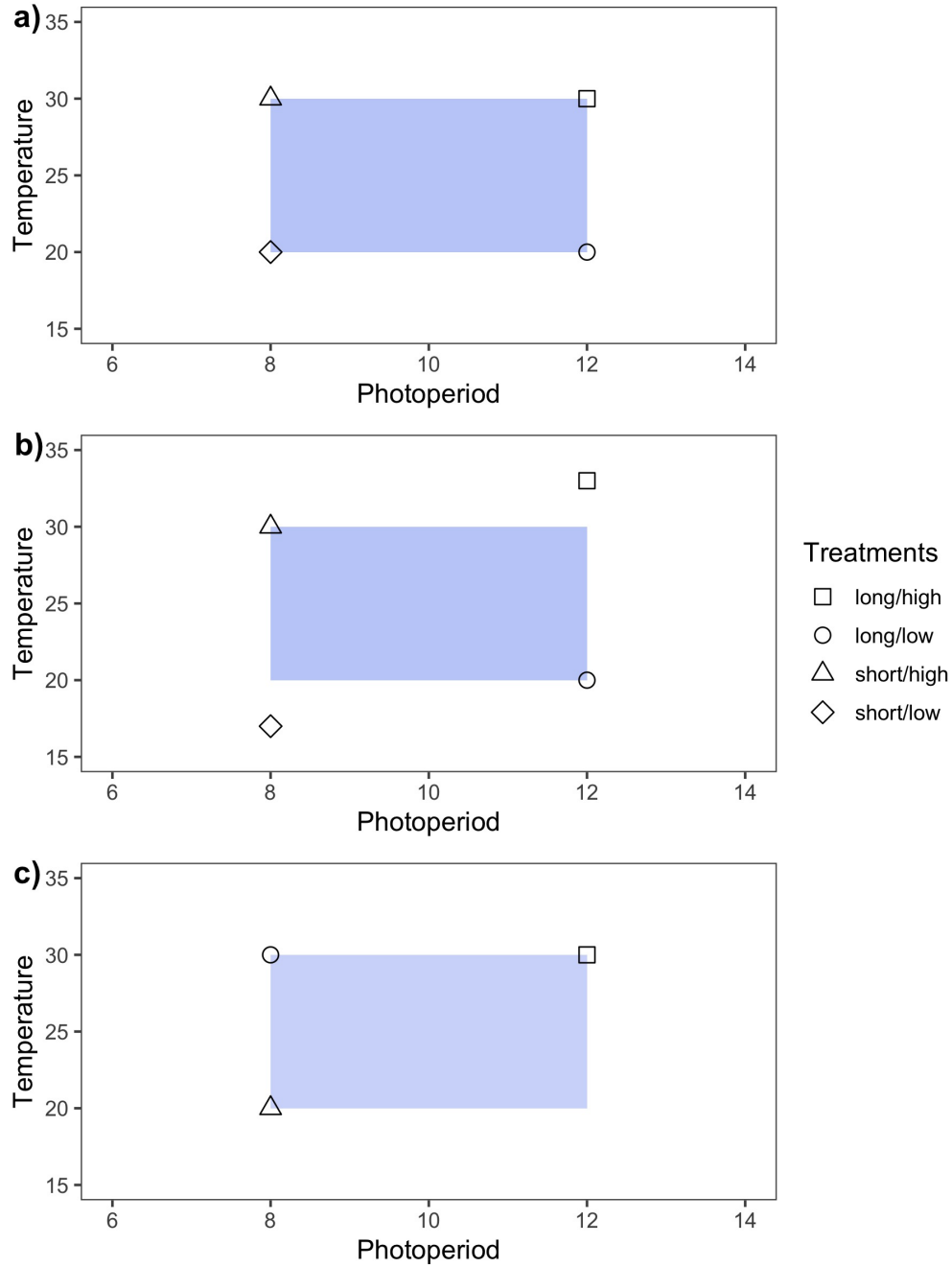


Figure 1: Idealized experimental designs demonstrate three approaches for varying temperature and light treatment level in controlled environment experiments. Design **a)** is fully factorial in that treatments levels are balanced and orthogonal. This design is appropriate for testing interactions between two or more variables. In **b)** the design is balanced both not orthogonal. Non-orthogonality in experiments often arises in experiments when there is covariation among the test variables is unaccounted for. In **c)**, the experimental design is orthogonal but unbalanced. Lack of balance in experiments often arises due to time, space or resource limitations.

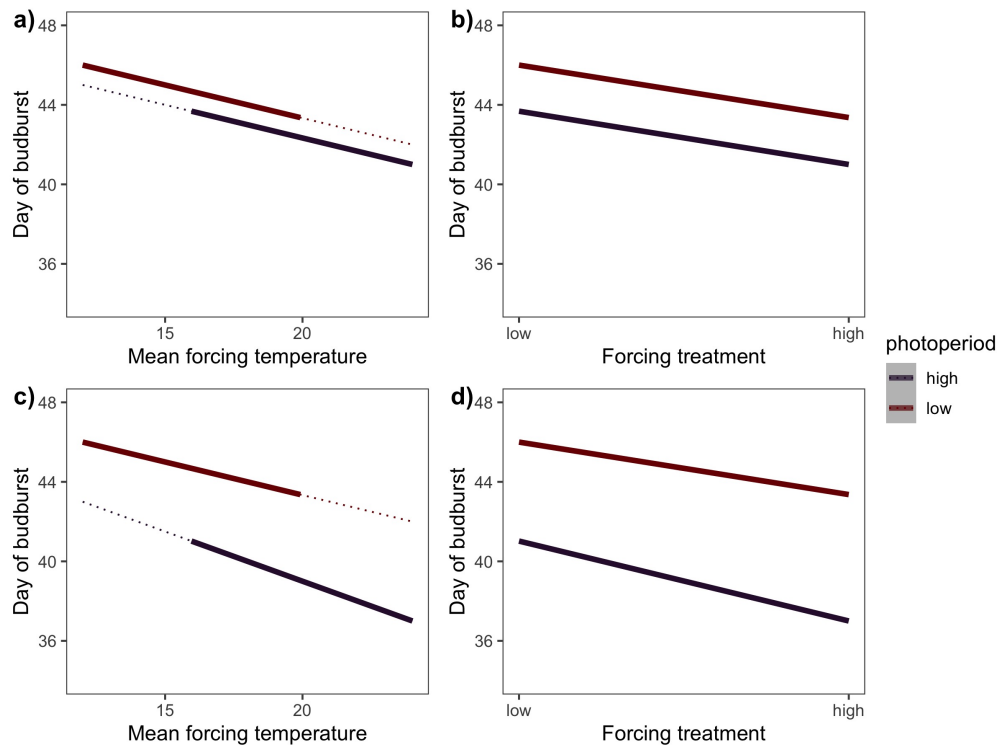


Figure 2: Estimated effects of photoperiod and forcing on phenology. a) True effects no interactions. b) estimated effects . c) True effects with interactions. d) estimated effects.

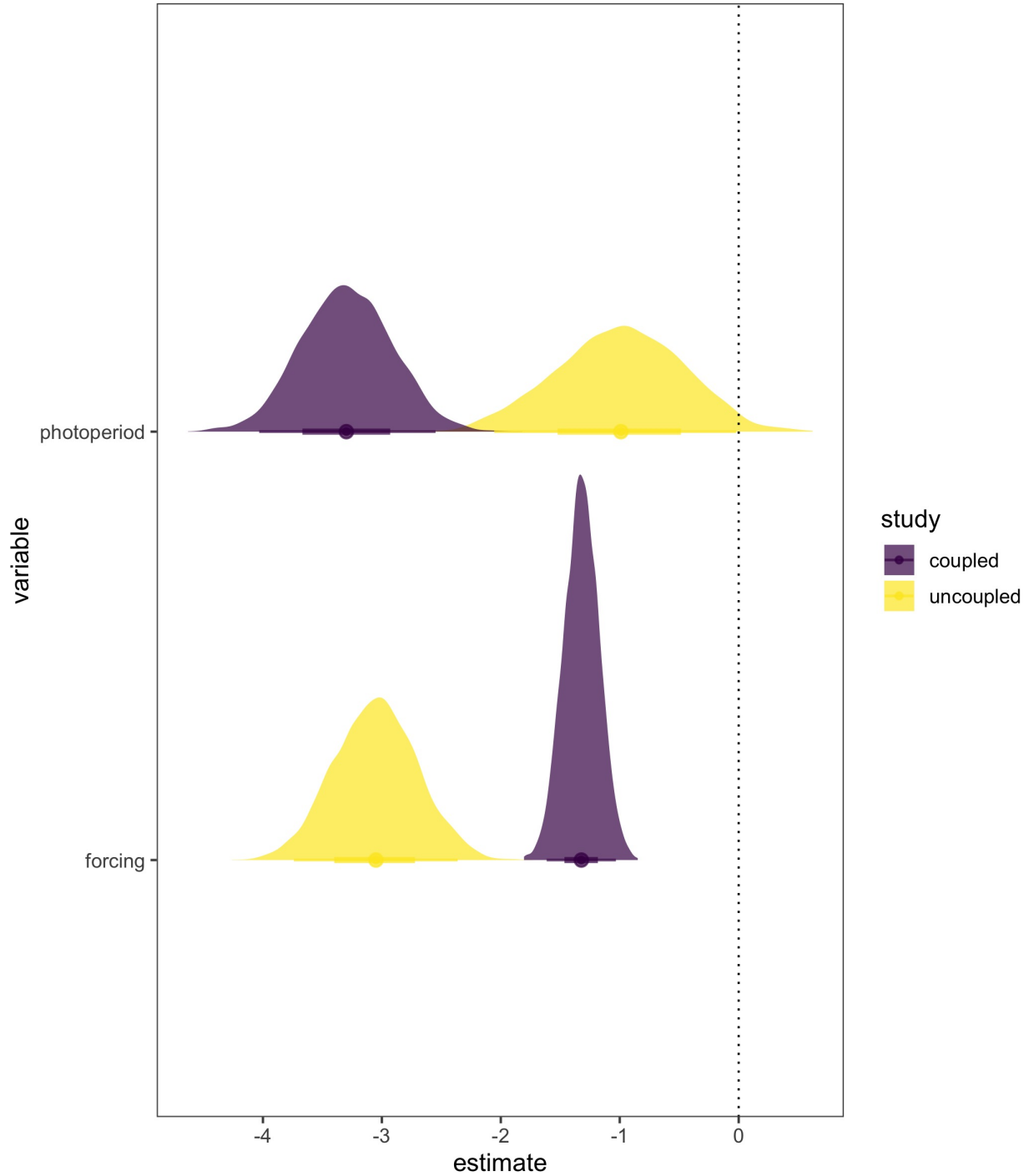


Figure 3: Estimated effects of one hour increase in photoperiod and one degree C increase in forcing on the day of leaf expansion using an alternative methods of varying thermo-period relative to photoperiod. Dots indicate the estimated mean effect, thick and thin bars the 50% and 97.5% credible intervals respectively. The full posterior distributions are also depicted for each parameter estimate. The colors represent the study design effect, with purple representing study design in which photo- and thermo-period co-vary, and yellow representing a design where there periodicity of each variable is independent of the other.

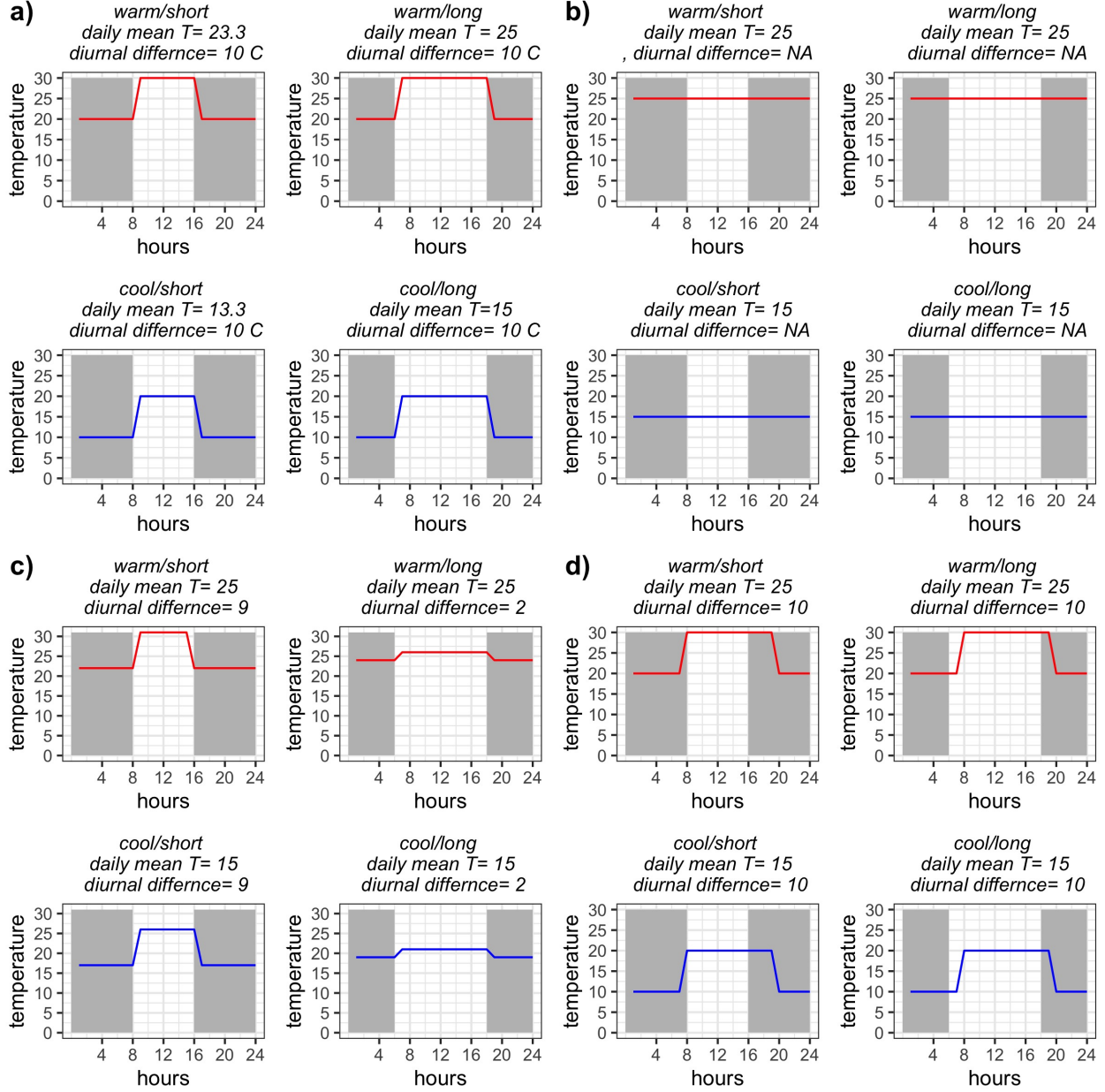


Figure 4: Conceptualized experimental designs to test temperature and daylength interactions on a biological response. In **a)** the design incorporates a standardize diurnal temperature fluctuation across all treatment. Because this thermoperiod is coupled with the photoperiod, while the same day and night temperatures are applied for the high and low temperature treatments respectively, the mean daily temperatures differ across each photoperiod treatment generating non-orthogonality. Designs **b),c)** and **d)** are all designs that can correct this non-orthogonality. Design **b)** manipulated temperature intensity only (no thermoperiodicity). In **c)** photo- and thermo- periods are still are coupled but the orthogonality of mean daily temperature is maintained by proportionately varying the diurnal temperature fluctuations across treatments. In design **d)** standard diurnal temperature fluctuations are maintained but, thermoperiod and photoperiod are decoupled and varied independently, maintaining orthogonality daily mean temperatures.