**3.3 MATERIALS AND METHODS**

**Study site.** The Forestry Research Centre of the Sabah Forestry Department is located in the Malaysian state of Sabah next to the Sepilok Forest Reserve (5° 10` N, 117° 56` E). The climate of Sabah is predominantly aseasonal, but there are relatively regular wetter and drier periods throughout the year (Maycock *et al.*, 2005; Born *et al.*, 2014; Margrove *et al.*, 2015). The mean annual rainfall is 3136 (±921 SD) mm with an average annual temperature of 27.3°C (as measured at the nearby Sandakan airport ca. 15 km away). The climate also varies supra-annually, linked to cyclical changes in the El Niño Southern Oscillation (ENSO), causing extremes of precipitation and drought (Margrove et al., 2015; Moerman et al., 2013; Slik, 2004).

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| **Figure 1 | The eight treatments of water-inundation durations over a single cycle.** Plants were inundated with water for different durations during the experiment (inundated for 0, 3, 6, 9, 12, 15, 18, 21 days per 21 days). One cycle lasted 21 days, and we drained all water bottles on the first day of a new cycle. Each cycle was repeated four times, and hence the experiment lasted 84 days. These eight levels were combined to produce a single continuous explanatory variable which we used to predict our various responses. |

**Experimental design.** We used a 375 m2 shade house (25 x 15 m) with a single shade cloth covering all sides to house the experiment (O’Brien, Philipson, Tay, & Hector, 2013; Philipson et al., 2014, 2012). Using an LAI-2000 plant canopy analyser (LI-COR, Nebraska, USA), we measured the reduction of photosynthetically active radiation under the shade cloth from full exposure. The percentage of direct sunlight under the shade cloth was similar to a large forest gap (22.4% ±1.8 SD; Philipson *et al.*, 2012). We choose a gap environment for our study as although they form a smaller component of the forest structure by area, gaps are pivotal in the growth and development of dipterocarps and are essential for these trees to reach sizes where they can become adults (Brown & Whitmore, 1992; Ghazoul, 2016). We partitioned the shade house into five blocks of 5 x 15m to control for unknown environmental and sampling effects. Within each block, 160 seedlings were randomly positioned and spaced at 0.5 m intervals ensuring independence.

Ten species of the Dipterocarpaceae family were used in this experiment to enable trait-based analysis in response to the treatments. Each species had 80 individual seedlings equitably distributed across the treatment gradient and between experimental blocks. Therefore, for each treatment level, there were ten individuals per species. We collected seedlings from 92 different mothers across four separate forest reserves in Sabah (Table S1). We included as many mothers from as many locations as possible to maximise genetic diversity (Cheng Choon et al., 2017). Mothers were distributed as evenly as possible throughout the experiment by block and treatment. Seeds were collected directly from mother trees during a general flowering event in 2014 using a big-shot catapult (SHERRILLtree, USA) and rope to shake branches and release seeds. We used wet jute sacks to germinate seeds and transplanted these into 9 x 15 cm polybags filled with a local alluvial soil and sawdust within 1 week. Seedlings grew inside a nursery for 16 months under a 91% shade cloth and with ambient watering. Six weeks before the start of the experiment, each seedling was transferred to a larger 15 x 20 cm polybag to ensure that the root growth was unrestricted during the experiment. We transferred the seedlings from the nursery into the experimental shade house and placed each one within their own independent 5 L plastic water bottle for six weeks of acclimation.

We designed a treatment gradient with eight different water-inundation duration levels. The gradient ranged from continuously to never inundated and levels equally distributed between 21 days (Fig. 1). We repeated this set of treatment durations four times during the experiment, and therefore the total period of experimentation was 84 days. We consider the eight durations of water-inundation to form a continuous explanatory variable. These eight water inundation treatments were designed to mimic the natural variation of water-logging cycles in the forest. Micro-topography, soil drainage and rainfall interact to control the duration of water-inundation. On inundated days of the cycle, silicone gel was applied to the floor of the water bottle to seal drainage holes in the base and we filled the bottle with water. An overflow hole prevented the water from reaching 2.5 cm above the soil line. On days where seedlings were to experience no effects of waterlogging, we removed the silicone gel, and the water was allowed to quickly drain.

**Measurements.** Our first question was, do species respond negatively to water-inundation episodes, and is there a difference between the species in their survival, growth and photosynthesis? These three responses were used to determine the tolerance of a plant to water-inundation. We classified dead seedlings as those with no green living tissue remaining under the bark at the end of the experiment. For growth, we measured seedling diameters at 5 cm above the soil line on the first day of the experiment with digital callipers, and at the end of every treatment cycle. Two measurements were made perpendicular to each other and the average taken by calculating the area of an ellipse and recalculating the diameter. The relative growth rate of each surviving seedling was calculated using the logarithm of diameter's difference over time (Sheil, Burslem, & Alder, 2008). We expressed the relative growth rates as mm mm-1 month-1­ (30 days). Photosynthesis and respiration were measured using an LCpro-SD advanced photosynthesis measurement system with the leaf chamber attachment (ADC Bioscientific ltd., Herts, UK). We measured randomly selected plants in the shade enclosure between 10:00 and 15:00 on 11 days during day 15-41 of the experiment. From this randomised sample, photosynthesis and respiration measurements were taken on 389 inundated and 160 non-inundated plants. Individuals were on average measured 3.57 times (SD ± 1.49). In total 548 measurements were made at ambient levels of CO2, temperature and PAR density. The average photo-synthetically active radiation on a leaf was 269.8 µmol m-2 s‑1 ± 9.53 SE. These four responses to our treatment were used to assess the effect of water-inundation on growth and survival variables.

Do species traits play a role in tolerance to water-inundation? We measured a suite of morphological traits, namely: wood density, stomatal index (SI), specific leaf mass (SLM), leaf mass fraction (LMF), root mass fraction (RMF), root air density and root wood density (Table S2). We measured all species trait values from plants taken from the nursery, not the experiment. Wood density was measured from a 3-5cm piece of wood from the base of the stem (n = 10, Table S2). This sample’s volume was measured using a balance and a pycnometer. The piece of wood was oven dried at 105°C for 48 hours and then weighed to calculate the dry mass per volume. To calculate root wood density, we sampled all roots around a central cross-section and used the same method as above (n = 10, Table S2). We measured three leaf traits: specific leaf mass, stomatal index, and leaf mass fraction (n = 10, Table S2). Leaf samples were dried in an oven at 60°C for 48 hours. Specific leaf mass was measured using a leaf disk on randomly selected fully developed mature leaves. A hole punch was used to extract each leaf disks taking care to avoid primary and secondary leaf veins. Disks were oven dried and weighed. Stomatal index is a dimensionless index that incorporates the size and density of stomata (Inoue, Kenzo, Tanaka-Oda, Yoneyama, & Ichie, 2015; Sack, Cowan, Jaikumar, & Holbrook, 2003). To calculate the stomatal index, take the product of the density of stomata and the guard cell length squared. Stomatal density and size influence the efficiency of water use and photosynthesis (Berryman, Eamus, & Duff, 1994). We applied clear nail varnish to the abaxial side of the leaf. When dry, a hole punch was used to cut a leaf disk with the coating. The varnish creates an impression of the leaf surface, which we transferred to a glass slide. A microscope with a video link was used to count the density of stomata and measure the length of guard cells (n = 6, Table S2).

For the leaf and root mass fraction calculations, we destructively harvested seedlings. The woody tissue was oven dried at 105°C for 48 hours whereas leafy tissue was dried at 60°C for 48 hours. After drying, we weighed all the plant material. The number of individuals harvested per species depended upon the available quantity (mean individuals per species was 19 ± 10 SD for a total of 194 individuals, see Table S2). We calculated both leaf and root biomass fractions as a proportion of the total biomass.

To calculate the root air-space, we measured the weight of a root sample in a pycnometer surrounded by water. The sample and water were then placed in a vacuum chamber for 24 hours at a ca. 98% vacuum, which forced water into the sample and pushed the air out. The treated sample was then placed inside the pycnometer again, filled with water, and reweighed. As the pycnometer has a constant volume, we can measure any additional water pressured into the sample, and calculate the difference. This difference reflects the volume of root air-space within root tissue.

**Statistics.** We used linear mixed effects models (LMMs) and generalised linear mixed effects models (GLMMs) to analyses the data directly collected from the nursery experiment. A GLMM was used to model survival with a binomial error distribution, and LMMs were used to model relative growth rate (mm mm-1 month-1, 30 day month) and photosynthesis (µmol m-2 sec-1) with a Gaussian distribution. In all three models, we modelled the fixed effects in the same way – the continuous response as a function of water-inundation duration, species identity and initial seedling diameter. Log transformations were considered for diameter and inundation duration to improve the model fit. We fit a maximal model with all interactions and used the dredge function to calculate the AIC for all model combinations. We defined the best model as the simplest model within 4 AIC points of the minimum AIC calculated – thereby ensuring parsimony (Barton, 2016). We found that all residuals had equal variance, an expectation of zero, and followed a Gaussian distribution. For the survival model, the random effects structure was: block (5 levels), and mother (92 levels). The growth model random effect structure included block, mother and census interval (4 levels). The random effect structure of the photosynthesis model was block, mother, and day of measurement (11 levels). Random effects all explained greater than zero variance and therefore kept in the model. ANOVA type III was used to calculate explained variance, with the Satterthwaite approximation to calculate the degrees of freedom.

We investigated the association between species responses to water-inundation duration. Using the three mixed effects models, we extracted ten slopes for each species. Each coefficient represents a species’ tolerance (steeper slopes mean less tolerant) to water-inundation duration. We also extracted the species intercepts for the photosynthetic response, which estimates non-inundated rates of carbon intake. These models were bootstrapped to calculate the standard deviation for these coefficients. We tested three models, growth rates predicting survival, photosynthesis rates predicting survival and photosynthesis rates predicting growth. Each model’s equation was constructed in this format because of considerations of the chain of events. For instance, if a plant is dead, then we cannot have any growth or photosynthesis, and therefore survival cannot predict growth or photosynthesis responses. This logic can also be applied to photosynthesis predicting growth, as growth can be thought of as a reaction to carbon intake, although there is an edge case whereby non-structural carbohydrates could provide resources for growth without photosynthesis. Each model was weighted by the inverse of the predictors’ standard deviations, to give less weight to more uncertain values. All models met the required assumptions.

We tested whether seedling growth and survival were dependent on maintaining carbon intake. Our response variable was the net photosynthetic rate (µmol m-2 s‑1), which we calculated by subtracting respiration from photosynthesis. We used a linear mixed effects model with a Gaussian distribution, to model the response as an interaction between species and treatment, plus the diameter. We tested the variables with a log transformation in case the relationship was non-linear, and used AIC to select the most parsimonious model. Random effects in this model were block, mother, and day of measurement. Using the linear equations, we calculated the carbon compensation point of each species. This point is where photosynthetic gains and respiratory loss are equal. Using this variable, we predicted the survival and growth response of the species with a univariate linear model. We find the residuals support the assumptions of this linear random effect and linear model.

We investigated seven functional traits as predictors of species' differential responses in growth, survival, photosynthesis, and photosynthetic compensation point of inundated plants. For each trait measured we calculated the species mean, and standard deviation (wood density, specific leaf mass (SLM), stomatal index (SI), root mass fraction (RMF), leaf mass fraction (LMF), root air density, and root wood density). We predicted the responses to the treatment with each trait using univariate linear models, weighted by the inverse of the standard deviation of the focal trait of that model. We only implement univariate analyses as exploration showed additional variables and interactions were not robust due to a lack of degrees of freedom which would likely lead to spurious results. One trait, SLM, had an extreme outlier (*S. xanthophylla*) that was 6.12 standard deviates higher than the mean of the other nine species. Although we have no reason to doubt the validity of this value, it violated linear model assumptions in all cases. Although this species’ SLM value affected model outcomes, it did not change the broader interpretation of our analysis. Therefore, we removed this species from the SLM analysis. All 21 models were validated using residual plots, which showed, normality, independence, equality of variance, and with an expectation of zero. Due to the exploratory nature of the functional trait analyses, we corrected for all non-independent p-values using the Bonferroni adjustment.

R 3.3.3 was used to analyse these data (<http://r-project.org>).

Barton, K. (2016). Multi-Model Inference. *CRAN Repository*, (1), 1–63.

Berryman, C. A., Eamus, D., & Duff, G. A. (1994). Stomatal responses to a range of variables in two tropical tree species grown with CO2 enrichment. *Journal of Experimental Botany*, *45*(274), 539–546.

Born, J., Pluess, A. R., Burslem, D. F. R. P., Nilus, R., Maycock, C. R., & Ghazoul, J. (2014). Differing life history characteristics support coexistence of tree soil generalist and specialist species in tropical rain forests. *Biotropica*, *46*(1), 58–68. https://doi.org/10.1111/btp.12083

Brown, N. D., & Whitmore, T. C. (1992). Do dipterocarp seedlings really partition tropical rain forest gaps? *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, *335*(1275), 369–378. https://doi.org/10.1098/rstb.1992.0028

Cheng Choon, A., O’Brien, M. J., Ng, K., Lee, P., Hector, A., Schmid, B., & Shimizu, K. (2017). Genetic diversity of two tropical tree species of the Dipterocarpaceae following logging and restoration in Borneo: high genetic diversity in plots with high species diversity. *Plant Ecology and Diversity*, *9*(5–6), 459–469. https://doi.org/10.1080/17550874.2016.1270363

Ghazoul, J. (2016). *Dipterocarp biology, ecology, and conservation*. Oxford Uni. Press, Oxford, UK.

Inoue, Y., Kenzo, T., Tanaka-Oda, A., Yoneyama, A., & Ichie, T. (2015). Leaf water use in heterobaric and homobaric leafed canopy tree species in a Malaysian tropical rain forest. *Photosynthetica*, *53*(2), 177–186. https://doi.org/10.1007/s11099-015-0105-6

Margrove, J. A., Burslem, D. F. R. P., Ghazoul, J., Khoo, E., Kettle, C. J., & Maycock, C. R. (2015). Impacts of an extreme precipitation event on dipterocarp mortality and habitat filtering in a Bornean tropical rain forest. *Biotropica*, *47*(1), 66–76. https://doi.org/10.1111/btp.12189

Maycock, C. R., Thewlis, R. N., Ghazoul, J., Nilus, R., & Burslem, D. F. R. P. (2005). Reproduction of dipterocarps during low intensity masting events in a Bornean rain forest. *Journal of Vegetation Science*, *16*(6), 635–646. https://doi.org/10.1111/j.1654-1103.2005.tb02406.x

Moerman, J. W., Cobb, K. M., Adkins, J. F., Sodemann, H., Clark, B., & Tuen, A. A. (2013). Diurnal to interannual rainfall δ18O variations in northern Borneo driven by regional hydrology. *Earth and Planetary Science Letters*, *369*–*370*, 108–119. https://doi.org/10.1016/j.epsl.2013.03.014

O’Brien, M. J., Philipson, C. D., Tay, J., & Hector, A. (2013). The influence of variable rainfall frequency on germination and early growth of shade-tolerant dipterocarp seedlings in Borneo. *PLoS ONE*, *8*(7), 1–9. https://doi.org/10.1371/journal.pone.0070287

Philipson, C. D., Dent, D. H., O’Brien, M. J., Chamagne, J., Dzulkifli, D., Nilus, R., … Hector, A. (2014). A trait-based trade-off between growth and mortality: evidence from 15 tropical tree species using size-specific relative growth rates. *Ecology and Evolution*, *4*(18), 3675–3688. https://doi.org/10.1002/ece3.1186

Philipson, C. D., Saner, P., Marthews, T. R., Nilus, R., Reynolds, G., Turnbull, L. A., & Hector, A. (2012). Light-based regeneration niches: evidence from 21 dipterocarp species using size-specific RGRs. *Biotropica*, *44*(5), 627–636. https://doi.org/10.1111/j.1744-7429.2011.00833.x

Sack, L., Cowan, P. D., Jaikumar, N., & Holbrook, N. M. (2003). The ‘hydrology’ of leaves: co-ordination of structure and function in temperate woody species. *Plant, Cell and Environment*, *26*(8), 1343–1356. https://doi.org/10.1046/j.0016-8025.2003.01058.x

Sheil, D., Burslem, D. F. R. P., & Alder, D. (2008). The interpretation and misinterpretation of mortality rate measures. *Journal of Ecology*, *83*(2), 331–333.

Slik, J. W. F. (2004). El Niño droughts and their effects on tree species composition and diversity in tropical rain forests. *Oecologia*, *141*(1), 114–120. https://doi.org/10.1007/s00442-004-1635-y