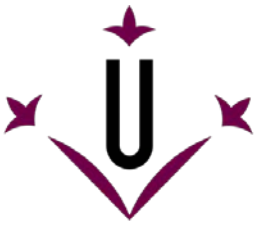

Assessment of intraspecies variability in fungal growth initiation of *Aspergillus flavus* and aflatoxin B_1 production under static and changing temperature levels using different initial conidial inoculum levels

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Abstract

Intraspecies variability in fungal growth and mycotoxin production has important implications for food safety. Using the Bioscreen C we have examined spectrophotometrically intraspecies variability of *A. flavus* using 10 isolates under different environments, including temperature shifts, in terms of growth and aflatoxin B_1 (AFB_1) production. Five high and five low AFB_1 producers were examined. The study was conducted at 5 isothermal conditions (from 15 to 37 °C) and 4 dynamic scenarios (between 15 and 30 °C). The experiments were carried out in a semisolid YES medium at 0.92 a_w and two inoculum levels, 10^2 and 10^3 spores/mL. The Time to Detection (TTD) of growth initiation was determined and modelled as a function of temperature through a polynomial equation and the model was used to predict TTD under temperature upshifts conditions using a novel approach. The results obtained in this study have shown that a model can be developed to describe the effect of temperature upshifts on the TTD for all the studied isolates and inoculum levels. Isolate variability increased as the growth conditions became more stressful and with a lower inoculum level. Inoculum level affected the intraspecies variability but not the repeatability of the experiments. In dynamic conditions, isolate responses depended both on the temperature shift and, predominantly, the final temperature level. AFB_1 production was highly variable among the isolates and greatly depended on temperature (optimum temperature at 30-35 °C) and inoculum levels, with often higher production with lower inoculum. This suggests that, from an ecological point of view, the potential isolate variability and interaction with dynamic conditions should be taken into account in developing strategies to control growth and predicting mycotoxin risks by mycotoxigenic fungi.

Keywords fungi; mycotoxins; modelling; inoculum size

1. Introduction

Fungal growth and mycotoxin contamination of food products represent an important food safety issue for the food industry. *Aspergillus* species are particularly important because some of them are xerophilic and able to colonise a range of food matrices, resulting in spoilage problems and mycotoxin contamination. This causes significant economic losses of staple food crops. Many factors can influence fungal growth in food products including nutritional composition, temperature, pH, water activity (a_w), atmospheric composition, presence and concentration of preservatives, different fungal communities, as well as storage times. Inter- and intra-species differences have been shown to be an important source of variability in terms of fungal growth and mycotoxin production (Abbas et al., 2005; Astoreca et al., 2007; Belli et al., 2004; Garcia et al., 2011a, 2011b; Lahouar et al., 2016; Romero et al., 2007; Santos et al., 2002; Singh et al., 2015; Yogendrarajah et al., 2016).

Usually, spoilage by filamentous fungi is visible to the naked eye in the form of characteristic colonies on the surface of food products, especially bakery goods. In general, spoilage has been evaluated by physically measuring the rate of colonisation on the food surface. However, the assessment of filamentous fungal activity is complex because they grow in three dimensions and are able to colonise a greater substrate surface area than yeasts or bacteria (Dantigny et al., 2005). The measurement of hyphal extension rates, usually reported as radial growth rate, is probably the simplest and most direct method to measure fungal growth. Nevertheless, as stated by Medina et al. (2012), these measurements do not account for the true representation of the three-dimensional nature of fungal growth, although there is a relationship between radial growth and fungal biomass (Trinci, 1971). In addition, the whole process is time consuming and requires significant inputs of time and consumables. Methods based on spectrophotometry (turbidimetric measurements) have been widely used for bacterial growth but rarely for examining ecophysiology of filamentous fungi.

74 Spectrophotometric assays provide fast results that are expressed in Optical Density (O.D.)
75 units. Only a few authors have used this kind of approach for mycological studies (Medina et
76 al., 2012; Mohale et al., 2013; Rossi-Rodrigues et al., 2009; Samsudin et al., 2016). The use of a
77 semi-solid agar medium has been effectively utilised to examine relative growth in relation to
78 environmental factors and also in relation to different anti-fungal compounds (Medina et al.,
79 2012).

80 There is interest in understanding the relationship between initial inoculum size and how this
81 affects the relative growth rate in relation to environmental conditions (Aldars-García et al.,
82 2016; Baert et al., 2008; Barberis et al., 2012; Burgain et al., 2013; Garcia et al., 2010; Gougouli
83 et al., 2011; Morales et al., 2008). In addition, how does initial inoculum size affect the capacity
84 for mycotoxin production? This could be important as the initial inoculum load in a food matrix
85 may influence, or indeed determine, how much mycotoxin is produced. In this study, we used
86 *Aspergillus flavus* as the target mycotoxigenic species because it colonises a range of cereals,
87 nuts and spices and contaminates them with aflatoxins, especially aflatoxin B_1 (AFB_1) which is a
88 class 1A carcinogen (IARC, 1993).

89 In nature, the fungal community consists of a range of species as well as isolates of the same
90 species. It is important to try and model the effects of ecophysiological factors on the activity
91 of mycotoxigenic species, especially with differing inoculum loads. There have been few
92 studies which have focused on different inoculum sizes of isolates of the same species in
93 relation to steady state temperatures and temporal shifts in this factor.

94 Thus, the objectives of this study were to (i) compare the impact of different steady state
95 temperatures (15-35 °C) on relative initial growth of 10 isolates of *A. flavus* at two initial
96 inoculum levels (10^3 and 10^2 spore/mL); (ii) examine the effect of four temperature shifts
97 (between 15-30 °C) and inoculum size on rates of growth using the Bioscreen C; (iii) evaluate
98 the effect of initial inoculum size and steady state and temperature shifts on AFB_1 production

and (iv) examine what impact these environmental conditions/shifts have on within-isolate variation using a secondary metabolite conducive medium. The ten tested isolates were divided into two groups, based on their AFB_1 production ability: 5 high and five low AFB_1 producers.

2. Material and methods

2.1. *A. flavus* isolates

Ten isolates of *A. flavus* isolated from chilli powder, maize grains and pistachio nuts were used in this study (Table 1). Isolates were categorized as "low AF producer" if AFB1 levels were between LOD and 100 ng/g agar and "high AF producer" for isolates that produced concentrations higher than 100 ng/g agar. There were five high and five low AFB_1 producers.

2.2. Inoculum preparation, culture medium and inoculation

Isolates were sub-cultured on Malt Extract Agar (Sigma-Aldrich, Dorset, UK) at 25 °C for 7 days to obtain heavily sporulating cultures. After incubation, spores were collected by scraping the surface of the plates, diluting them in sterile water adjusted to 0.92 a_w with glycerol containing Tween 80 (0.05% v/v), and filtered through sterile glass wool into a tube. The total spore concentrations (per ml) were determined and decimal dilutions (in sterile water adjusted to 0.92 a_w with glycerol, containing Tween 80 (0.05% v/v)), were prepared to obtain the two different spore concentrations: 10^5 and 10^4 spores/mL for each isolate. The basic medium used in this study was a semisolid Yeast Extract Sucrose (YES) agar, whose protocol of preparation was previously optimised by Medina et al. (2012) and adjusted to 0.92 a_w. Spore suspensions were used to inoculate semi-solid YES medium. 100 µL of the spore suspension was pipetted into 9.9 mL of semi-solid YES agar, for each spore concentration, resulting in two final

concentrations of 10^3 and 10^2 spore/mL in the semisolid YES agar were prepared for each isolate.

2.3. Growth assessment

Growth was studied at 15, 20, 25, 30, 35, 37 °C and four temperature shift treatments from 15 to 25 °C (F1), 15 to 30 °C (F2), 20 to 25 °C (F3) and 20 to 30 °C (F4), after 48 hours at the lowest temperature.

Optical densities, which are directly related to the fungal biomass of *A. flavus* (Medina et al.,2012) were recorded using a Bioscreen C Microbiological Growth Analyser (Labsystems, Helsinki, Finland). 100-well microtitre plates specifically manufactured for this machine were loaded with the 10^3 and 10^2 spore/mL semisolid YES agar of each isolate; one plate per spore concentration was used. The wells of the microplate were filled with 300 µL of the inoculated medium, thus ca. 300 and 30 spores were inoculated in each well for the 10^3 and 10^2 spores/mL inocula, respectively. For each temperature condition two inoculum levels were set with 9 replicates (well) per isolate (10 isolates x 9 replicates=90 wells plus 10 empty wells). Overall, one plate per inoculum level and temperature condition was used.

The O.D. was recorded every 30 min using the 600nm filter over a 7 days period, except for 20 °C and 15 to 25 °C (F1), where 14 and 9 days respectively, were needed to reach the growth threshold set for the experiments. Data were recorded using the software Easy Bioscreen Experiment (EZExperiment) provided by the manufacturer and then exported to a Microsoft® Excel® Professional 2010 (14.0.4756.1000) (Microsoft Corporation, Redmond, Washington, USA) sheet for further analysis.

2.4. Aflatoxin B_1 assessment

145 Following incubation, the well contents was decanted in order to analyse the mycotoxin
146 concentration. AFB_1 extraction was carried out as follows: the contents of 3 wells was collected
147 for each isolate and temperature condition, in triplicate. AFB_1 was extracted with 0.8 mL of
148 chloroform, shaken for 1 min and left stationary for 20 min. The chloroform phase was
149 separated and the aqueous phase re-extracted twice with 0.8 mL of chloroform. The organic
150 extracts were combined and evaporated to dryness. The residues were derivatized using
151 trifluoroacetic acid as described by the AOAC (2000) and transferred to a HPLC vial. All
152 derivatized samples were analysed by HPLC (Agilent 1200 series HPLC (Agilent, Berkshire, UK)).
153 Chromatographic separations were performed on a stainless steel C18 column (Phenomenex
154 Luna ODS2 150 x 4.6 mm, 5 µm). Methanol: water: acetonitrile (30:60:10) was used as the
155 mobile phase at a flowrate of 1mL/min. AFB_1derivative fluorescence was recorded at
156 excitation and emission wavelengths of 360 and 440 nm respectively. Standard curves were
157 constructed with different levels of AFB_1. Aflatoxin B_1 recovery assays were performed to
158 ensure the analytical quality of the results. This showed that for all standards the recovery rate
159 was >87%.

160 2.5. Data analysis

161 2.5.1. Time to Detection (TTD) for static temperatures

162 Raw datasets obtained from the Bioscreen C were subjected to further analysis. Before
163 analyses, the average of the measurements for each well during the first 60 min was calculated
164 and automatically subtracted from all subsequent measurements in order to remove the
165 different signal backgrounds. Then, the TTD for an O.D. of 0.1 was obtained using a Microsoft®
166 Excel® template (kindly provided by Dr. R. Lambert), which used linear interpolation between
167 successive O.D. readings.

168 In order to stabilise the variance, for the TTD comparisons, a square root transformation was
169 used. However, raw data are presented in Tables and Figures. The Kruskal–Wallis test was used

170 to establish the differences among median TTD values of the 10 isolates under the different
171 treatment levels at $p<0.05$.

172 Finally, based on the TTD, a polynomial model that described the TTD as a function of the
173 temperature was fitted. The general expression of the polynomial model used was:

174 $$TTD = a_0 + a_1 T + a_2 T^2 \quad (1)$$

175 a_i are the constants to be estimated, T is the independent variable (temperature) and TTD is
176 the response variable. This model was chosen as only temperature levels in the range 15-37 °C
177 were tested, which were not enough to fit models used for the whole range of growth
178 temperatures.

179 Statistical analysis was carried out with Statgraphics® Centurion XVI.I (Statpoint, Inc.,
180 Maryland, USA).

181

182 2.5.2. Prediction of TTD under temperature upshifts

183 The TTDs obtained under the static temperatures were used to design the experiments for
184 estimating the TTDs under temperature upshifts conditions. The time-temperature scenarios
185 studied included a single abrupt shift from a low to an upper temperature. The TTD under
186 temperature upshifts was predicted through the model fitted at constant temperature, and
187 compared to the experimental results generated under the temperature changing scenarios
188 (experimental TTD). These experiments were carried out in the same way as for the static
189 conditions.

190 The TTD of each isolate for the changing temperature (total TTD=TTDT) treatments consisted
191 of a single abrupt temperature shift from an initial temperature (TI) to a final, higher
192 temperature (TF) at a time ts. Thus, Fig. 1 was calculated using the following equation:

193 $$TTDT = \begin{cases} TTDI & \text{if ts} > TTDI \\ TTDF + (TTDI - TTDF) * \left(\frac{TF-TI}{TF}\right) * \left(\frac{ts}{TTDF}\right) & \text{if ts} < TTDI \end{cases} \quad (2)$$

194 Where TTDI and TTDF were calculated by substituting the corresponding temperature in the
195 polynomial equation (Eq. (1)). That is, when the temperature shift occurs before the end of
196 TTDI, after TTDF is consumed, the remaining TTD is a percentage of the interval (TTDI-TTDF),
197 which was assumed to be proportional to (i) the temperature shift, and (ii) the timing of the
198 temperature shift.

199 **3. Results**

200 3.1. Time to Detection (TTD) at constant temperature conditions: isolate variability and
201 inoculum size effects

202 In the first part of the study, the objective was to determine the influence of temperature on
203 the relative initial growth (time for an increase of 0.1 in the O.D.) of the 10 *A. flavus* isolates
204 and address their differences. Thus, TTDs of 0.1, indicating initial growth of the spores, were
205 calculated. In this study, all experiments were carried out at constant temperature conditions.
206 TTDs were further fitted to a second order polynomial equation (Eq. (1)) in order to predict the
207 TTD as a function of the temperature for each isolate. The developed polynomial models
208 accurately fitted the influence of temperature on the TTD, with goodness of fit (r^2) ranging
209 from 0.980 to 0.999. Both temperature and its quadratic term had significant effects on TTD
210 (Table 2).

211 All *A. flavus* isolates grew under all steady state temperature profiles tested, except for 15 °C.
212 According to the Kruskal-Wallis test, there were significant differences ($p<0.05$) in TTD among
213 isolates. However, these differences depended on the temperature profile tested. TTD values
214 followed the time sequence 37 <35 <30 <25 <20 °C in all cases. This pattern is shown in Fig. 2.
215 In general, all isolates showed similar TTD values at the same temperature and inoculum level.

The TTDs at 30, 35 and 37 °C did not revealed significant differences between isolates, at the 95% confidence level.

At 25 °C, differences between the 10 isolates increased (higher coefficient of variation (CV %)) which could suggest more within-isolate variability as temperature approached the lower limit for growth. Isolate responses at 20 °C were highly variable, not only among isolates but also within replicates of the same isolate. Furthermore at 20 °C in some replicates, conidia failed to initiate growth at all. At the five steady state temperatures, the isolate UdL-TA 3.327 had the lowest TTDs, and isolates UdL-TA 3.244 and UdL-TA 3.332 the highest. No differences in growth pattern were found between the low and high AFB_1 producer groups.

Statistical analysis ($p < 0.05$) showed a clear difference between the TTD for the two initial inoculum levels for all temperatures examined (Fig. 2). In addition, differences among isolates' TTD increased for the lower initial inoculum size (10^2 spores /mL), i.e., the difference in time was greater at the lower inoculum level. The differences between the 2 inoculum levels were greater at 20 °C. For example, for isolates UdL-TA 3.270 and UdL-TA 3.332, the differences at 30 °C were 0.7 and 4 hours for the high and the low inoculum level treatments respectively. At 20 °C these differences were 19 and 22 hours, respectively, for the low and the high inoculum levels. From Figure 2 it was also observed that the TTD was significantly affected by the spore concentration, and these differences were more marked as conditions approached the limit for conidial germination at low temperature. In general, within-isolate variability was more affected by the lower temperatures (20 °C) than by the inoculum level. Increasing the conidial inoculum size from 10^2 to 10^3 spores/mL, when temperature was 20°C (a realistic practical storage temperature for agricultural products), had a significant effect on the ability *of A. flavus* to initiate growth. At this temperature, a difference in the prediction of growth initiation of more than 2 days occurred depending on the initial inoculum level.

241 3.2. Prediction of relative initial growth times (TTD) as affected by temperature shifts

242 Generally, under ambient transport conditions temperature is not a fixed value and fluctuates
243 during distribution and the length of the food chain. Thus, it is important to better understand
244 the effect of such fluctuations on fungal growth due to temperature or indeed inoculum size
245 impacts on relative level of risk from growth or toxin contamination. Thus, an approach to
246 predict the effect of temperature shifts on the ability of fungi to grow (in our case by means of
247 the TTD) was developed taking into account the results from the static temperature studies.

248 At the same temperature profile and inoculum concentration level the 10 tested isolates did
249 not differ much in their TTDs. TTDs followed the time sequence F4<F2<F3<F1 except for isolate
250 UdL-TA 3.327 which was F4<F3<F2<F1 for both inoculum levels (Fig. 3). The highest difference
251 among isolates was observed in the 20 to 25 °C temperature regimes. The most difficult
252 temperature shift to initiate growth was from 15 to 25 °C, which took approximately 168 and
253 142 hours to reach the TTD, for the low (ca. 30 spores) and higher inoculum (300 spores)
254 concentration, respectively. However, the differences observed in this temperature profile (F1)
255 were more marked within isolate than among isolates (see large error bars). The standard
256 deviation was always greater for those profiles where the initial temperature was 15 °C.
257 Results for isolate UdL-TA 3.244 at 10^2 spores/mL are summarised in Figure 4 which represents
258 the TTD for the four shifting temperature scenarios and TTD of the static temperatures
259 involved in the dynamic profiles.

260 In order to evaluate the suitability of the model to predict the TTD of *A. flavus* conidial
261 germination and growth under changing temperature scenarios, the predicted TTDs derived
262 from equation 2 were compared to experimental TTDs obtained by carrying out the
263 experiment at set shifting temperature profiles.

264 The four temperature shifts were imposed prior to the TTD. Agreement between the model
265 predictions and experimental data results was assessed by plotting predicted TTD versus

observed TTD (Fig. 5). The observed response values agreed well with the predicted response values except for the temperature shift from 15 to 30 °C where a clear overestimation was obtained (approx. 50 hours).

Nonetheless, accurate predictions were possible under the two profiles with 25 °C as the final temperature, with a mean underestimation of only 4 hours made. Also, good agreement between the experimental and predicted TTD for an initial temperature of 20 °C and a final temperature of 30 °C (Fig. 5) was obtained, with a mean underestimation of 9 hours.

3.3. Effect of inoculum level, temperature profiles and intra-species variability on aflatoxin B_1 production

The amount of AFB_1 produced after 7 days' growth (except for 20 °C and 15 to 25 °C which were 14 and 9 days respectively due to the slow growth rates) was determined for all isolates and both inoculum levels (Fig. 6). Firstly, AFB_1 was not detected at 15 °C, when tested as a single constant temperature treatment. The amount of AFB_1 production depended on the temperature, and followed the profile 35>30>25>37>20 °C, for the higher inoculum level and the profile 35>30>25>20>37 °C, for the lower inoculum level. For some isolates the optimum production was at 30 °C, for some others at 35 °C.

The inoculum level had different effects on the AFB_1 production depending on the temperature. AFB_1 production was slightly higher at the higher initial inoculum level, except for the 37 °C steady state treatment, and most temperature shifts. This later pattern was more marked for the high AFB_1 producer isolates. For example, at 37 °C, isolate UdL-TA 3.270 produced 282.97 and 40.45 ng/g for the 10^2 and 10^3 spores/mL inocula, respectively, or to a lesser extent isolate UdL-TA 3.268 which produced 86.45 and 1.44 ng/g for the 10^2 and 10^3 spores/mL inocula, respectively. The same pattern was clearly found in the temperature shift from 20 to 30 °C. At 35, 30 and 25 °C, no significant differences were observed among inocula.

Only at the constant temperature of 20 °C was more AFB_1 produced at the higher inoculum level for all isolates. However, some isolates were not able to produce the toxin at this temperature.

Looking at the low producing isolates, no AFB_1 was produced at 35 °C, and very low amounts at 37 °C. However, at 25 °C some isolates of this group produced more AFB_1 than the ones in the high producer group, namely isolates UdL-TA 3.267, UdL-TA 3.269 and UdL-TA 3.325 at both initial inoculum levels. A similar behaviour was found at 20 °C where isolates UdL-TA 3.325, and UdL-TA 3.269, and the UdL-TA 3.325 for the 10^2 and 10^3 spores/mL inocula respectively, produced more AFB_1 than some isolates of the high producer group at the same inoculum level. As the groups were allocated based on production on PDA at 25 °C, it is clear that the differences in medium composition played an important role in AFB_1 production.

In order to determine the effect of temperature shifts, the theoretical amount of AFB_1 produced taking into account the time periods at each temperature was calculated as follows:

$$Calculated\ AFB_{1TI} = \frac{(AFB_{1TI} * ts) + (AFB_{1TF} * tf)}{ts + tf} \quad (3)$$

Where AFB_{1TI} and AFB_{1TF} correspond to the amount of toxin produced at the initial and final temperature, respectively, under the single steady state scenarios, and ts and tf are the time periods for the initial and final temperature respectively for each shifting temperature profile. Table 3 shows both theoretical and experimental AFB_1 concentrations.

No clear pattern was found where the shifting temperature treatments were used. For the changing scenario F1 (15 to 25 °C) a dramatic increase in AFB_1 production was detected, compared to the low levels detected at 15 and 25 °C, under steady state conditions. The same trend was observed for the shift from 20 to 30 °C, where in general a 3-4 fold increase in AFB_1 production was observed, when compared to the calculated AFB_1. In fact, the production under this condition was as higher as that observed at constant 35 °C, and much higher than

315 that observed at constant 30 °C. Thus in this case the stress imposed at 20 °C, followed to a
316 shift to a near to optimal temperature had a great impact. In contrast, the shift from 20 to 25
317 °C resulted in a lower AFB_1 production regarding when compared with that produced in the
318 steady state same two temperatures treatments. More variability was found for the shift from
319 15 to 30 °C, where for some isolates there was an overestimation and for others an
320 underestimation of AFB_1 production.

321 **4. Discussion**

322 4.1. Intraspecies variability for growth and AFB_1 production

323 Generally, food products are stored at suboptimal conditions to minimise mould growth and
324 this may influence the intraspecies variability in both germination and initial colonisation and
325 potential for mycotoxin production (Astoreca et al., 2007; Bellí et al., 2004; Garcia et al.,
326 2011a, 2011b; Lahouar et al., 2016; Mohale et al., 2013; Mugrabi de Kuppler et al., 2011; Pardo
327 et al., 2004; Parra and Magan, 2004; Romero et al., 2007; Tassou et al., 2009; Tauk-Tornisielo
328 et al., 2007; Yogendrarajah et al., 2016).

329 This study has utilised a suboptimal a_w level, representing environmental stress, to examine
330 and quantify effects of steady state and shifting temperatures on growth of groups of high and
331 low AFB_1 producing *A. flavus* isolates for the first time. The parameter studied was the TTD,
332 which is the time in which mould growth is detected at a certain biomass level as
333 demonstrated previously by Medina et al. (2012). These values are a very good approximation
334 to understand the growth of fungal colonies in a 3D space and at very low biomass levels. Due
335 to the use of a synthetic liquid media the amount of aflatoxins determined cannot be
336 extrapolated to real food substrates. However, the effect of intraspecies differences,
337 temperature and inoculum size can still be assessed, and it is an easy methodology to generate
338 data as starting point to build models for dynamic temperature profiles. Thus if a full model
339 was developed and applied to realistic food products, thorough validation would be necessary.

340 The present study has shown that it is possible to predict TTD under steady state and some
341 shifting temperatures. The results obtained in the first part of the study (steady state
342 temperatures) showed that as temperature approached the lower limit for conidial
343 germination and mycelial growth, intraspecies variability increased. This trend has been
344 observed using other criteria by some authors for other fungi. For example, Romero et al.
345 (2007) evaluated the effects of a_w (0.80-0.95) and temperature (15-35 °C) on lag phase of four
346 *Aspergillus carbonarius* isolates, and found the greatest difference at limiting conditions.
347 Garcia et al. (2011a) working with 79 isolates of *Penicillium expansum* reported coefficients of
348 variation for the lag phase of 12.7 and 14.3% at 20 and 1 °C, respectively. This suggests that
349 intraspecies variability is dependent on the environmental conditions, and is higher when
350 conditions are closer to the boundaries for activity. In the present study, we have focused on
351 steady state temperatures and shifting temperatures but under a fixed water stress condition.
352 In this situation, under lower temperature conditions isolate variability was found to be higher.

353 For shifting temperature scenarios, intraspecies variability did not appear to be significant,
354 with only a few isolates, among the 10 studied, behaving differently from the others. The final
355 temperature had the major effect on intraspecies variability. Within isolate variability was
356 more affected by the initial temperature than by the final temperature, since %CV was higher
357 for those profiles which were set initially at 15 °C.

358 Longer TTDs were observed when low inoculum levels were used. Conceptually, TTD should
359 parallel lag phases for the pool of spores prior to growth. Of course, the latter parameter has
360 been studied many times under different inoculum levels (Aldars-García et al., 2016; Baert et
361 al., 2008; Burgain et al., 2013; Chulze et al., 1999; Morales et al., 2008; Sautour et al., 2003).
362 Such studies have shown that changes in the inoculum size affected and increased the length
363 of the lag phases prior to growth when the inoculum size decreased. Moreover, the inoculum

364 level was also found to be a critical factor in TTD intraspecies variability: as inoculum
365 decreased, intraspecies variability increased.

366 Considering the effect of temperature on AFB_1 production, the amount produced was highly
367 variable among the 10 isolates. Production in *A. flavus* is highly variable and depends on
368 genotype, substrate and geographic origin, climate change and agronomic practice (Perrone et
369 al. 2014). Santos et al. (2002) studied the production of patulin and citrinin by 10 isolates of *P.*
370 *expansum*, and showed that patulin production was isolate dependent. Aldars-García et al.
371 (2015) predicted the probability of growth and AFB_1 production of *A. flavus* using a cocktail
372 inoculum of 25 isolates and an inoculum with a single isolate. Different results in terms of
373 growth behaviour were obtained for both inocula but not for AFB_1 production which gave very
374 similar probabilities, highlighting the possibility of a homogeneous boundary of AFB_1
375 production among isolates, although the amount produced by isolates was different. The
376 variability in the amount of mycotoxin produced will be influenced by nutritional substrate,
377 interacting environmental conditions, source, age and whether wild or sub-cultured on rich
378 artificial laboratory media (Garcia et al., 2011a; Romero et al., 2010; Yogendrarajah et al.,
379 2016).

380 There was not a clear effect of inoculum size on AFB_1 production but, interestingly, the
381 optimum temperature for AFB_1 production was at 30-35 ºC, lower than that for growth, as has
382 been shown for other toxigenic species.

383

384 4.2. Predicting relative initial growth (TTD) and AFB_1 production under shifting temperature
385 scenarios

386 In order to measure the effect of a temperature shift on the time needed to initiate growth, *A.*
387 *flavus* isolates were subjected to sudden temperature upshifts. Adaptation to environmental
388 stresses is usually explained by biological mechanisms in the cell, which requires a certain

389 amount of time depending on the cells' physiological state and the new environmental
390 conditions (Brooks et al., 2011; Swinnen et al., 2005). Many studies have been carried out
391 under fluctuating temperatures for bacterial pathogens (Bovill et al., 2000, 2001; Kim et al.,
392 2008; Koseki and Nonaka, 2012; Muñoz-Cuevas et al., 2010; Zwietering et al., 1994) and to a
393 lesser extent for fungi (Aldars-García et al., 2015; Gougouli and Koutsoumanis, 2012, 2010). In
394 some of them when models included germination or growth rates, instantaneously adaptation
395 to the new environment was assumed for these rates (Gougouli and Koutsoumanis, 2012) and
396 in other cases, when primary observations were modelled, for example, visible growth,
397 inclusion of a 'memory parameter' in the models was required for acceptable predictions
398 (Aldars-García et al., 2015).

399 Muñoz-Cuevas et al. (2010) developed a dynamic growth model for a *Listeria monocytogenes*
400 isolate. They found that growth behaviour depended not only on the magnitude of the change
401 between the previous and current environmental conditions but also on the current growth
402 conditions. Similarly, we found that TTD under dynamic temperature depended mainly on the
403 final temperature and to a lesser extent on the magnitude of the change and initial
404 temperature. Figure 4 illustrates an example of this dependence for isolate UdL-TA 3.244 at
405 10^2 spores/mL, in which all TTD for the changing temperature scenarios are around the TTD at
406 25 °C. This behaviour may suggest that 48 hours at a restrictive temperature could not be
407 enough to slow the cells' metabolism to a point which prevents them from quickly adapting to
408 better growth conditions.

409 In the present study, a model was developed for the prediction of the TTD under the changing
410 temperature scenarios. The model correctly predicted three of the four temperature
411 scenarios. At the 15 to 30 °C condition TTD was overestimated by the model, which may mean
412 that the time at 15 °C limited more than expected the later growth at 30 °C, which should have
413 been optimum. The stress imposed at 15 °C for the first 48 hours and the later adaptation to

414 the new favourable temperature is difficult to be reflected by the model. This kind of effect
415 needs to be studied in depth (including up and downshifts in temperature) to, for example,
416 include in the predictive model another parameter that accounts for the time to get adapted
417 to the new different temperature when there is a change from a really unfavourable
418 temperature for growth, 15 °C, to an optimal temperature for growth, such as 30 °C.

419 AFB_1 production under dynamic temperature conditions was enhanced under some scenarios
420 and inhibited under others. As a general rule, AFB_1 production seemed to be triggered by
421 wider shifts in temperature (increases of 10°C), while when the shifts were of 5 °C, the AFB_1
422 amounts produced were similar or lower than those expected from the results at constant
423 temperature. This reinforced the hypothesis that several kinds of stresses may trigger
424 mycotoxin production. Several authors have described that abiotic stress is involved in the
425 activation of mycotoxin biosynthetic genes (Jurado et al., 2008; Kohut et al., 2009; Schmidt-
426 Heydt et al., 2009). Then, the stress induced by the temperature shift may have a similar
427 effect, triggering AFB_1 production.

428 Garcia et al. (2012) studied mycotoxin production by *Fusarium* spp. under 3 changing
429 temperature scenarios (15 to 20 °C, 15 to 25 °C and 25 to 30 °C). They also found that for some
430 profiles the mycotoxin production was enhanced and for others inhibited. Furthermore, this
431 pattern was different depending on the mycotoxin studied. Ryu and Bullerman (1999) studied
432 the production of deoxynivalenol and zearalenone on rice with cycling temperatures, finding
433 that mycotoxin production was stimulated under the temperature shifts.

434 In our study, when comparing growth initiation and AFB_1 production, it was observed that
435 growth initiation occurred earlier with higher temperature (up to 37 °C), while the highest
436 AFB_1 production occurred at 35 °C, production being very low at 37 °C. Previous studies have
437 also shown a lower temperature for optimal AFB_1 production than for growth (Astoreca et al.
438 2012, 2014). Regarding dynamic scenarios, earlier growth was observed, as expected, when 30

°C was included in the profile and with higher temperature pairs; the same trend was observed for AFB_1 production, except for the upshift 20/25 for which production was lower than at 15/25; however, this may be attributed to a triggering of production at wider shifts in temperature, as pointed out before.

Although in our case, higher toxin production occurred with lower inoculum size, mainly under shifting temperature, but not at constant ones, published studies on the effect of inoculum revealed different outcomes on mycotoxin production. Direct relationship between the amount of mycotoxin produced and the inoculum size was reported by Aldars-García et al. (2016) and Chulze et al. (1999). On the other hand, Morales et al. (2008) reported that colonies from conidial suspensions of 10^6 spores/mL produced lower amounts of patulin (in apples) than those from the 10^4 spores/mL suspensions. These results may suggest a possible inhibition of germination, and thus mycotoxin production, when spore concentration is too high. Further research on this area is required to understand how inoculum size affects the biosynthesis of mycotoxins.

As conclusion, taking into account isolate variability and inoculum size in mycological studies would give more realistic results, since in a real scenario contamination we may encounter different isolates in a food product. Furthermore, it becomes evident that temperature shifts have an important effect on fungal behaviour, and that there is potential for modelling and predicting toxigenic mould behaviour under steady state and fluctuating temperatures. The data generated in the present study are useful for a better understanding of the behaviour of isolates under dynamic temperature scenarios, in order to improve our understanding of mycotoxin contamination of food matrices, and thus help in the development of approaches to improve shelf-life of products prone to fungal spoilage, improve shelf-life and improve food safety.

5. Acknowledgements

The authors are grateful to the Agència de Gestió d'Ajuts Universitaris i de Recerca de la Generalitat de Catalunya (AGAUR, grant 2014FI_B 00045), and to the Spanish Ministry of Economy and Competitiveness (MINECO, Project AGL2014-55379-P) for funding this work. The authors also would like to thank Dr. Ronald J.W. Lambert for kindly providing the TTD calculation Excel spreadsheet.

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629 Table 1. Description of the isolates used in the present study

Isolate code*	Origin	AFB_1 production on PDA at 25 °C for 7 days (ng/g)
UdL-TA 3.244	Chilli	20.5
UdL-TA 3.267	Pistachio nuts	25.9
UdL-TA 3.269	Pistachio nuts	28.3
UdL-TA 3.324	Maize grains	5.4
UdL-TA 3.325	Maize grains	1.5
UdL-TA 3.268	Pistachio nuts	471.2
UdL-TA 3.270	Pistachio nuts	114.8
UdL-TA 3.327	Maize grains	178.5
UdL-TA 3.331	Maize grains	547.2
UdL-TA 3.332	Maize grains	2114.6

630 *Isolate codes are the names of the cultures held in the Food Technology Department Culture Collection
631 of University of Lleida, Spain.

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639 Table 2. Parameter estimation of the polynomial models for TTDs (minutes) of the 10 *A. flavus* isolates at both inoculum levels.

Inoculum level	Variable	Coefficient estimated value [a]									
		UdL-TA 3.244	UdL-TA 3.267	UdL-TA 3.269	UdL-TA 3.324	UdL-TA 3.325	UdL-TA 3.268	UdL-TA 3.270	UdL-TA 3.327	UdL-TA 3.331	UdL-TA 3.332
10^2 spores/mL	Constant	77434± 5282	66100 ± 2336	75437± 3320	61534± 2424	65000± 2643	70988± 3070	64260± 3259	59395±1958	60100 ± 4172	67532±3684
	T	-4329± 374	-3643± 168	-4348± 240	-3372± 175	-3569± 191	-4096± 222	-3532±236	-3383±141	-3292±302	-3777±260
	T^2	63± 6	52± 2	65± 4	48 ± 3	51 ± 3	61 ± 3	51 ± 4	50 ± 2	47 ± 5	55 ± 4
	r^2	0.93	0.98	0.97	0.98	0.98	0.96	0.96	0.98	0.93	0.95
10^3 spores/mL	Constant	62490 ± 3171	51181± 2514	60660±2836	51837± 1772	52029 ± 2182	59022± 2360	54020± 2557	45936± 1254	48765± 2865	64245± 4101
	T	-3470± 228	-2774 ± 182	-3488± 205	-2842 ± 128	-2835± 158	-3398±171	-2964± 185	-2571± 90	-2643± 207	-3646± 295
	T^2	50± 3	39 ± 3	52 ± 3	41± 2	40± 2	51 ± 2	42 ± 3	38 ± 1	38 ± 3	54 ± 5
	r^2	0.96	0.96	0.96	0.98	0.98	0.97	96.87	0.99	0.95	0.94

640 [a] mean values ± sd

641 *All coefficient estimates were significant at $P < 0.05$

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643 Table 3. Experimental and calculated amount of AFB_1 produced by the 10 isolates tested (in ng/g in YES medium) at the four dynamic scenarios
644 assayed, and at both inoculum levels.

Dynamic temperature		15 to 25 °C		15 to 30 °C		20 to 25 °C		20 to 30 °C	
		Calculated AFB_1	Experimental AFB_1	Calculated AFB_1	Experimental AFB_1	Calculated AFB_1	Experimental AFB_1	Calculated AFB_1	Experimental AFB_1
10^3 spores/mL	UdL-TA 3.244	10.0	348.4	224.6	21.7	9.2	1.2	224.6	154.9
	UdL-TA 3.267	79.0	246.2	437.9	117.9	72.7	0.7	438.1	560.1
	UdL-TA 3.269	317.7	461.8	1227.6	394.2	296.0	6.1	1231.8	1506.6
	UdL-TA 3.324	0.5	71.8	3.7	0.9	0.7	0.1	4.0	52.9
	UdL-TA 3.325	147.4	1639.3	1434.1	242.7	145.9	41.9	1444.6	1967.9
	UdL-TA 3.268	27.3	904.7	797.6	320.6	27.8	32.2	800.3	3003.3
	UdL-TA 3.270	14.0	728.7	1052.7	2197.3	15.8	12.5	1055.7	3253.5
	UdL-TA 3.327	21.0	824.8	598.6	1467.8	26.8	21.6	606.1	3069.6
	UdL-TA 3.331	31.2	484.3	571.2	541.7	28.6	1.8	571.3	297.6
	UdL-TA 3.332	14.5	818.2	625.4	19.1	26.2	17.0	638.3	2759.8
10^2 spores/mL	UdL-TA 3.244	0.2	594.3	121.4	2.8	0.2	0.0	121.4	164.9
	UdL-TA 3.267	27.8	492.7	487.2	81.8	25.5	0.0	487.2	82.7
	UdL-TA 3.269	159.5	720.6	904.2	81.8	146.6	0.0	904.3	1041.6
	UdL-TA 3.324	3.4	117.1	19.6	3.4	3.1	0.0	19.6	11.7
	UdL-TA 3.325	5.3	4606.2	885.6	131.3	6.4	0.0	887.1	2023.2
	UdL-TA 3.268	20.2	944.3	826.2	1304.9	18.7	0.1	826.4	5820.1
	UdL-TA 3.270	4.5	0.0	1045.2	1195.5	4.2	0.0	1045.2	5811.2
	UdL-TA 3.327	13.6	696.7	672.7	1587.5	14.2	2.9	674.4	5930.6
	UdL-TA 3.331	18.0	612.2	727.4	1209.3	16.6	0.0	727.5	416.0
	UdL-TA 3.332	0.8	389.9	214.8	11.3	0.8	0.0	214.9	5571.2

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Figure 1. Schematic representation of TTD as affected by the temperature shift.

Figure 2. TTDs at 37, 35, 30, 25 and 20 °C for the ten isolates studied at a) 10^3 and b) 10^2 spores/mL. Error bars are standard deviations (n=9).

Figure 3. Experimental TTD obtained under the dynamic temperature profiles: 15 to 25 °C (black bars), 20 to 25 °C (dark grey bars), 15 to 30 °C (white bars) and 20 to 30 °C (light grey bars) for the a) 10^3 spores/mL inoculum and the b) 10^2 spores/mL inoculum for the 10 isolates tested. Error bars are standard deviations (n=9).

Figure 4. Comparison among the TTD under both static and dynamic temperature profiles for isolate UdL-TA 3.244 at 10^2 spores/mL.

Figure 5. Predicted TTD versus observed TTD at the four changing temperature for the ten isolates studied at a) 10^3 and b) 10^2 spores/mL and the four dynamic temperature profiles: 15 to 25 °C (■), 20 to 25 °C (×), 15 to 30 °C (◆) and 20 to 30 °C (▲).

Figure 6. Aflatoxin B1 production (ng/g) of 10 *A. flavus* isolates under a) five constant temperature levels and b) four dynamic scenarios at both inoculum levels.

Figure

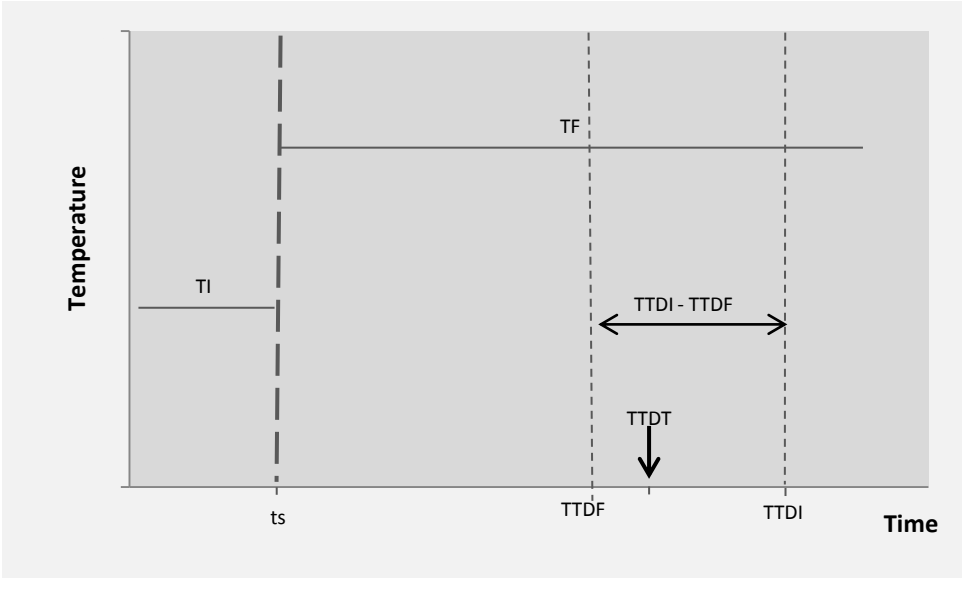
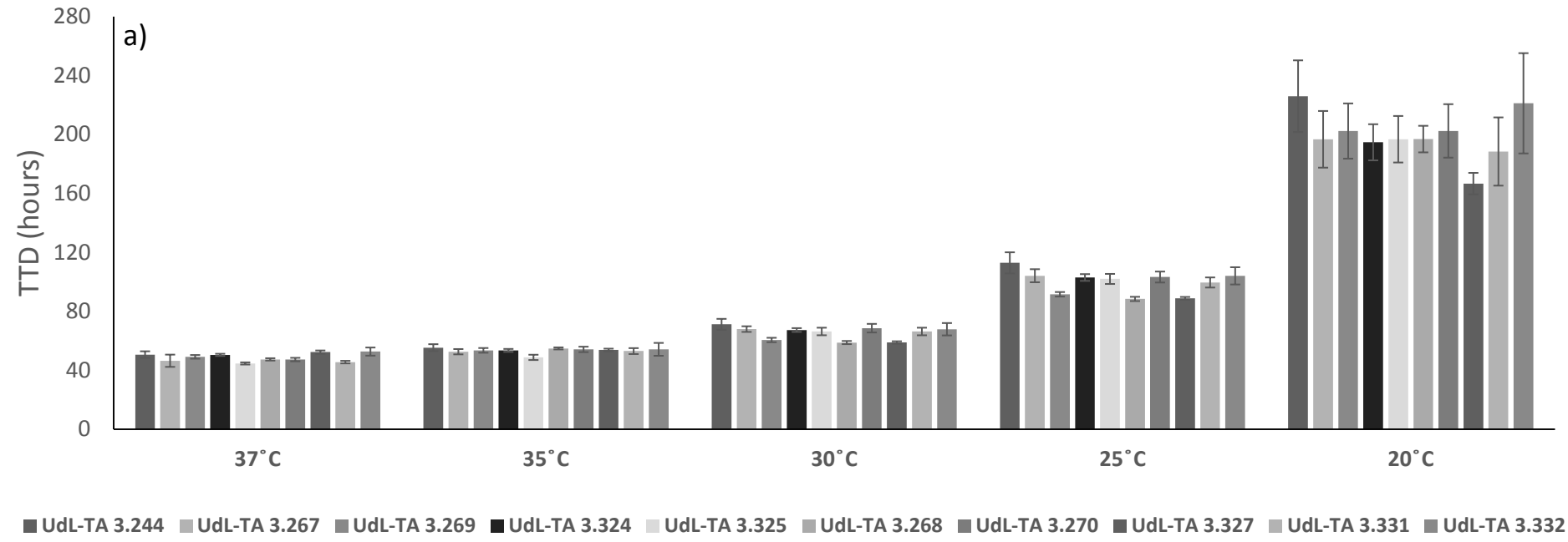


Figure2


a)
TTD (hours)
280
240
200
160
120
80
40
0
37˚C
35˚C
30˚C
25˚C
20˚C
UdL-TA 3.244
UdL-TA 3.267
UdL-TA 3.269
UdL-TA 3.324
UdL-TA 3.325
UdL-TA 3.268
UdL-TA 3.270
UdL-TA 3.327
UdL-TA 3.331
UdL-TA 3.332

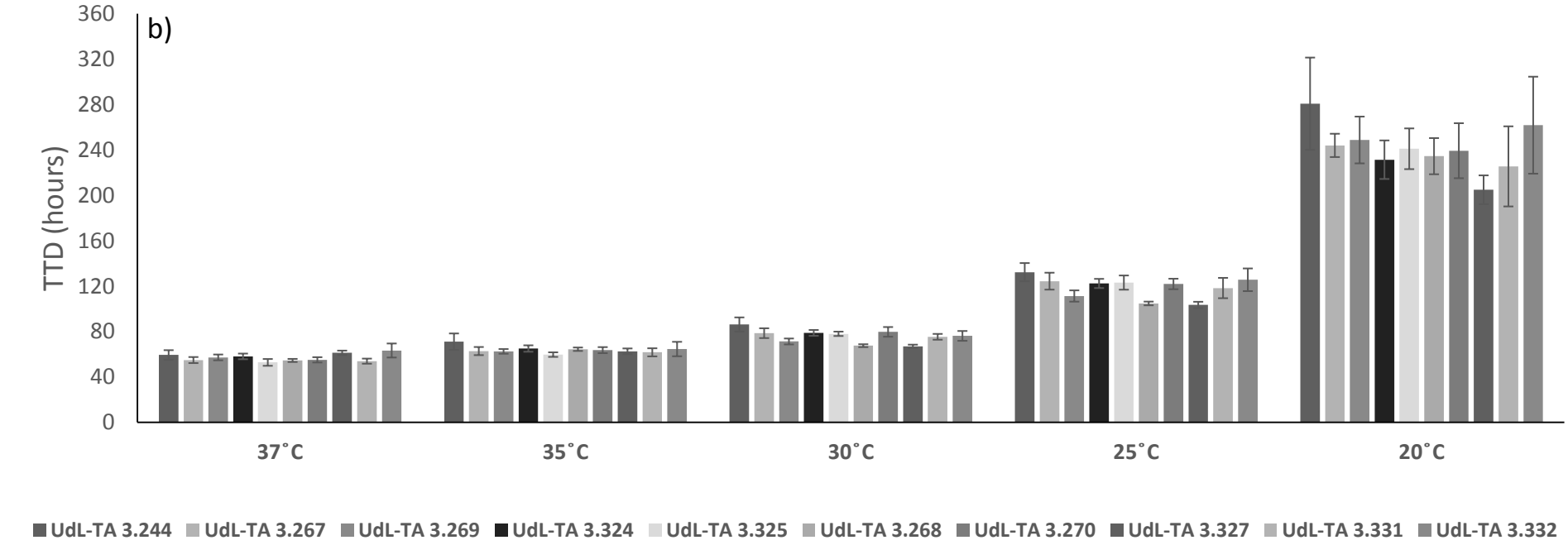
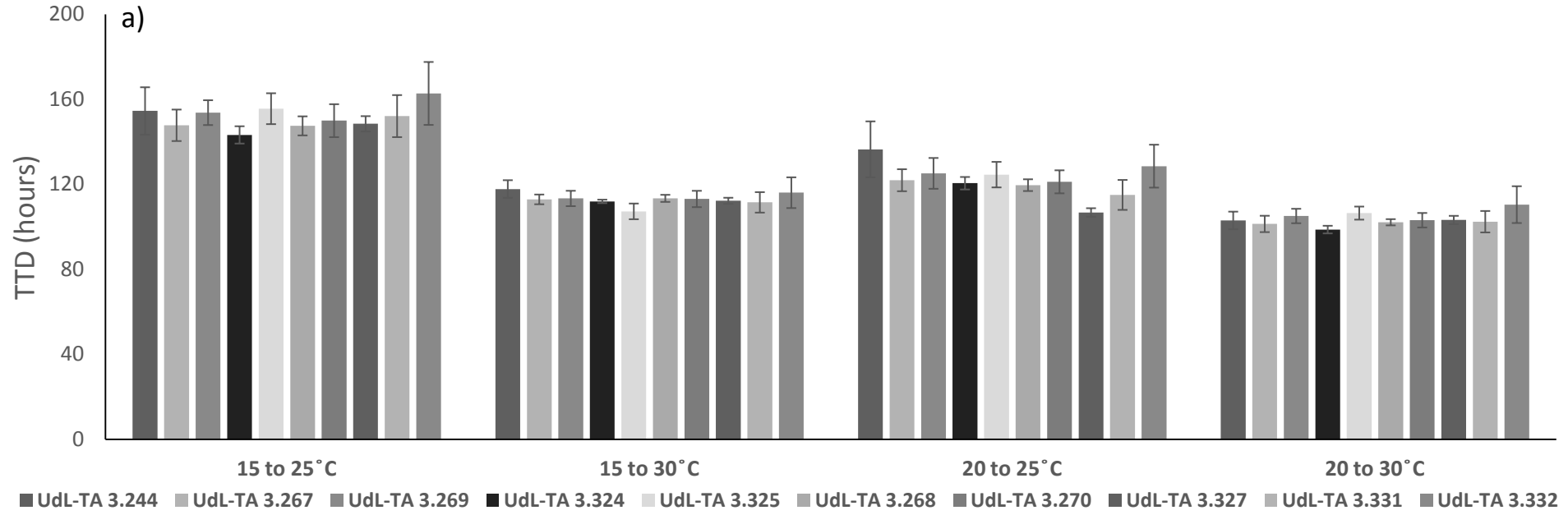

b)
TTD (hours)
360
320
280
240
200
160
120
80
40
0
37˚C
35˚C
30˚C
25˚C
20˚C
UdL-TA 3.244
UdL-TA 3.267
UdL-TA 3.269
UdL-TA 3.324
UdL-TA 3.325
UdL-TA 3.268
UdL-TA 3.270
UdL-TA 3.327
UdL-TA 3.331
UdL-TA 3.332

Figure3


a)
200
160
120
80
40
0
TTD (hours)
15 to 25˚C
15 to 30˚C
20 to 25˚C
20 to 30˚C
UdL-TA 3.244
UdL-TA 3.267
UdL-TA 3.269
UdL-TA 3.324
UdL-TA 3.325
UdL-TA 3.268
UdL-TA 3.270
UdL-TA 3.327
UdL-TA 3.331
UdL-TA 3.332

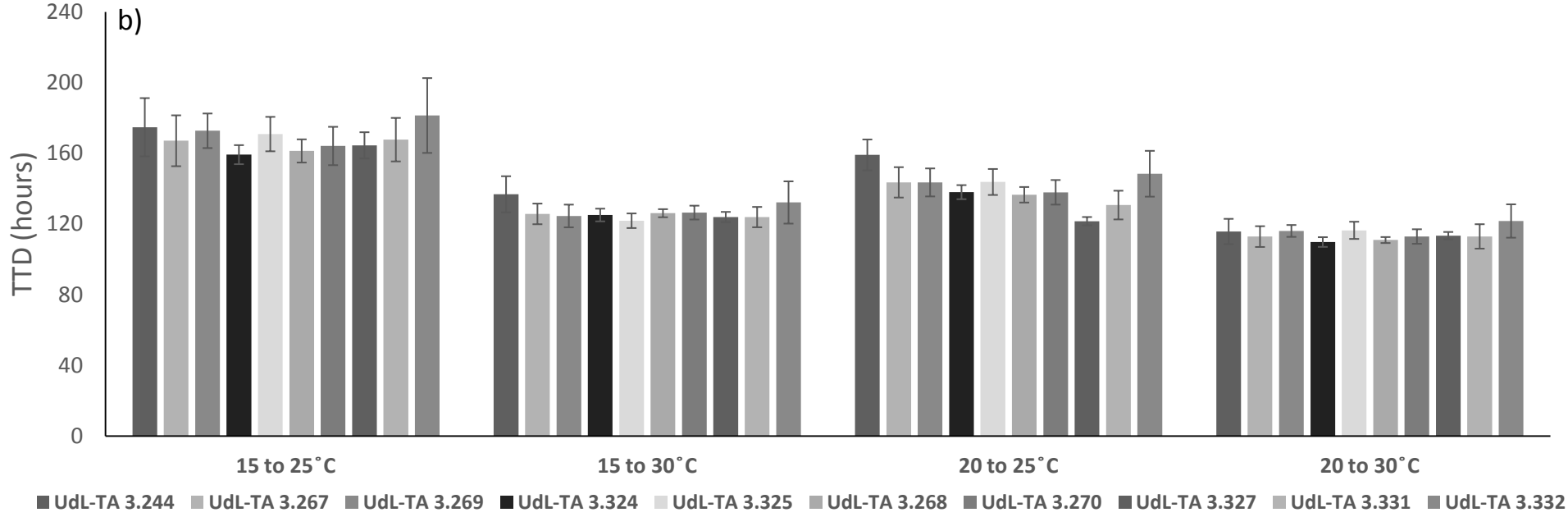

b)
240
200
160
120
80
40
0
TTD (hours)
15 to 25˚C
15 to 30˚C
20 to 25˚C
20 to 30˚C
UdL-TA 3.244
UdL-TA 3.267
UdL-TA 3.269
UdL-TA 3.324
UdL-TA 3.325
UdL-TA 3.268
UdL-TA 3.270
UdL-TA 3.327
UdL-TA 3.331
UdL-TA 3.332

Figure4

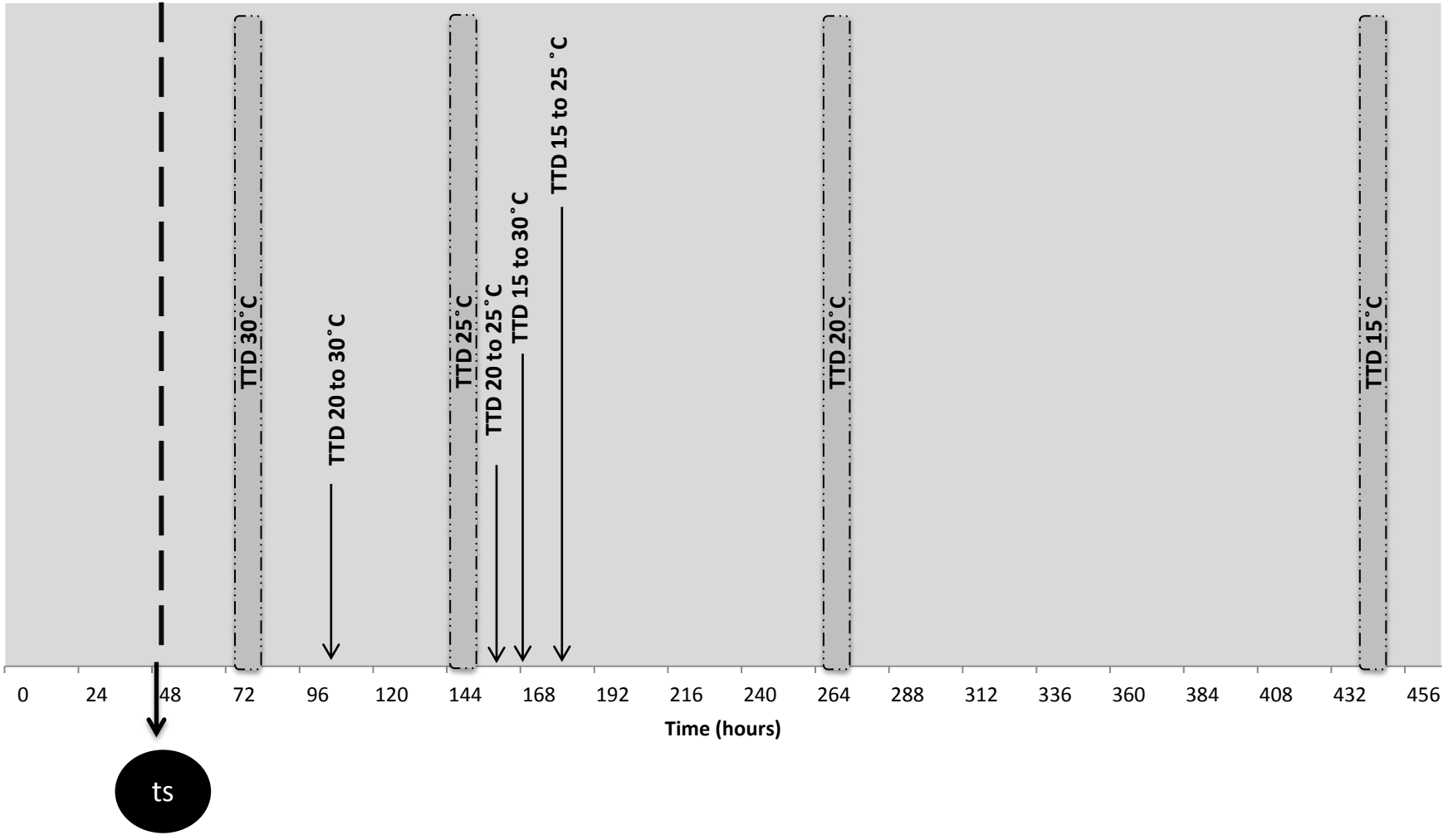
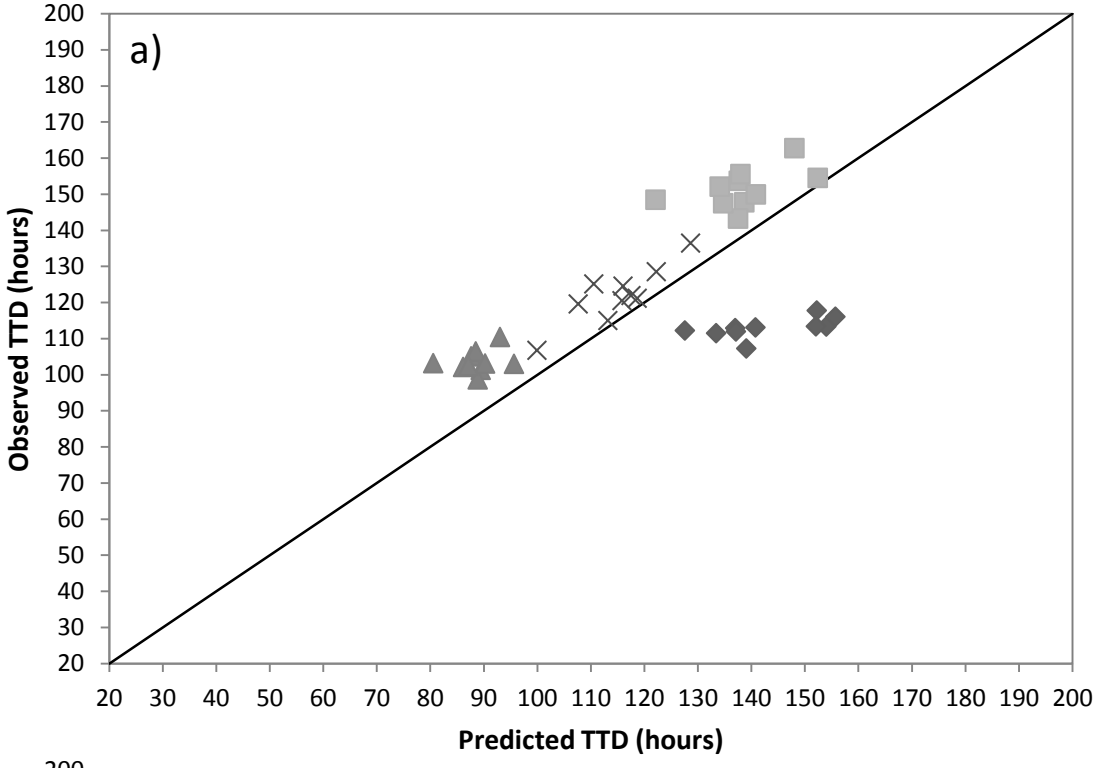

TTD 30˚C
TTD 20 to 30˚C
TTD 25˚C
TTD 20 to 25˚C
TTD 15 to 30˚C
TTD 15 to 25 ˚C
TTD 20˚C
TTD 15˚C
0
24
48
72
96
120
144
168
192
216
240
264
288
312
336
360
384
408
432
456
Time (hours)
ts

Figure5


a)
Observed TTD (hours)
Predicted TTD (hours)
20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180 190 200

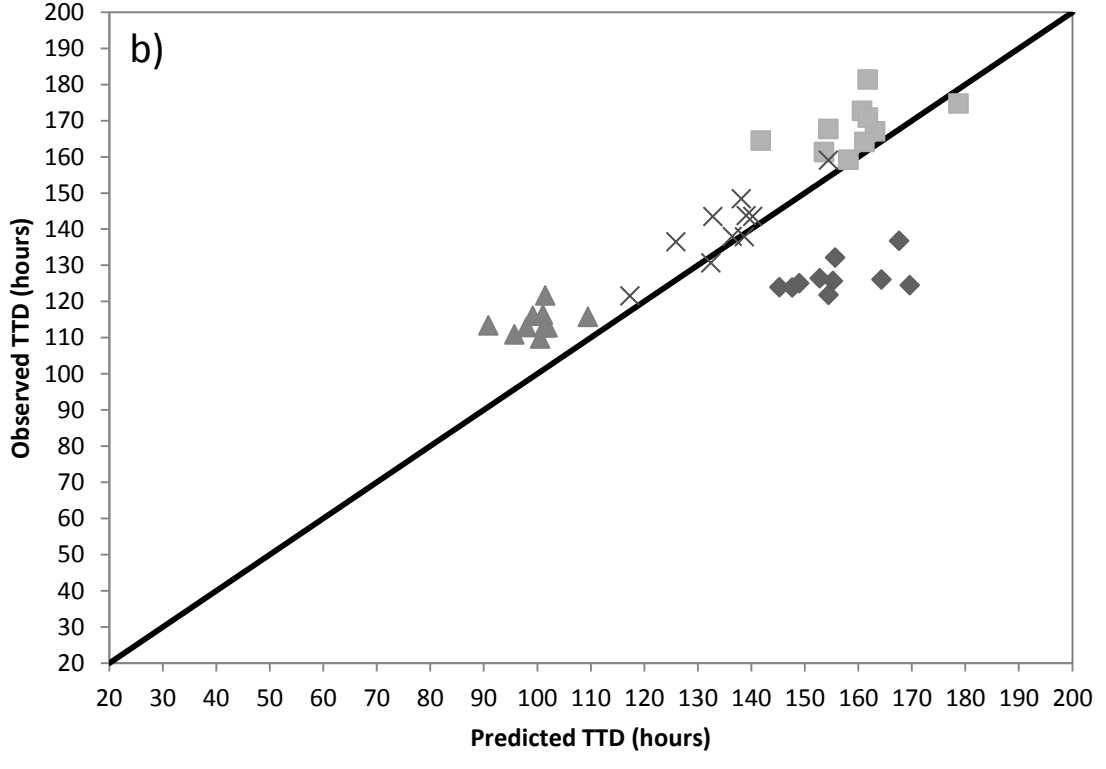
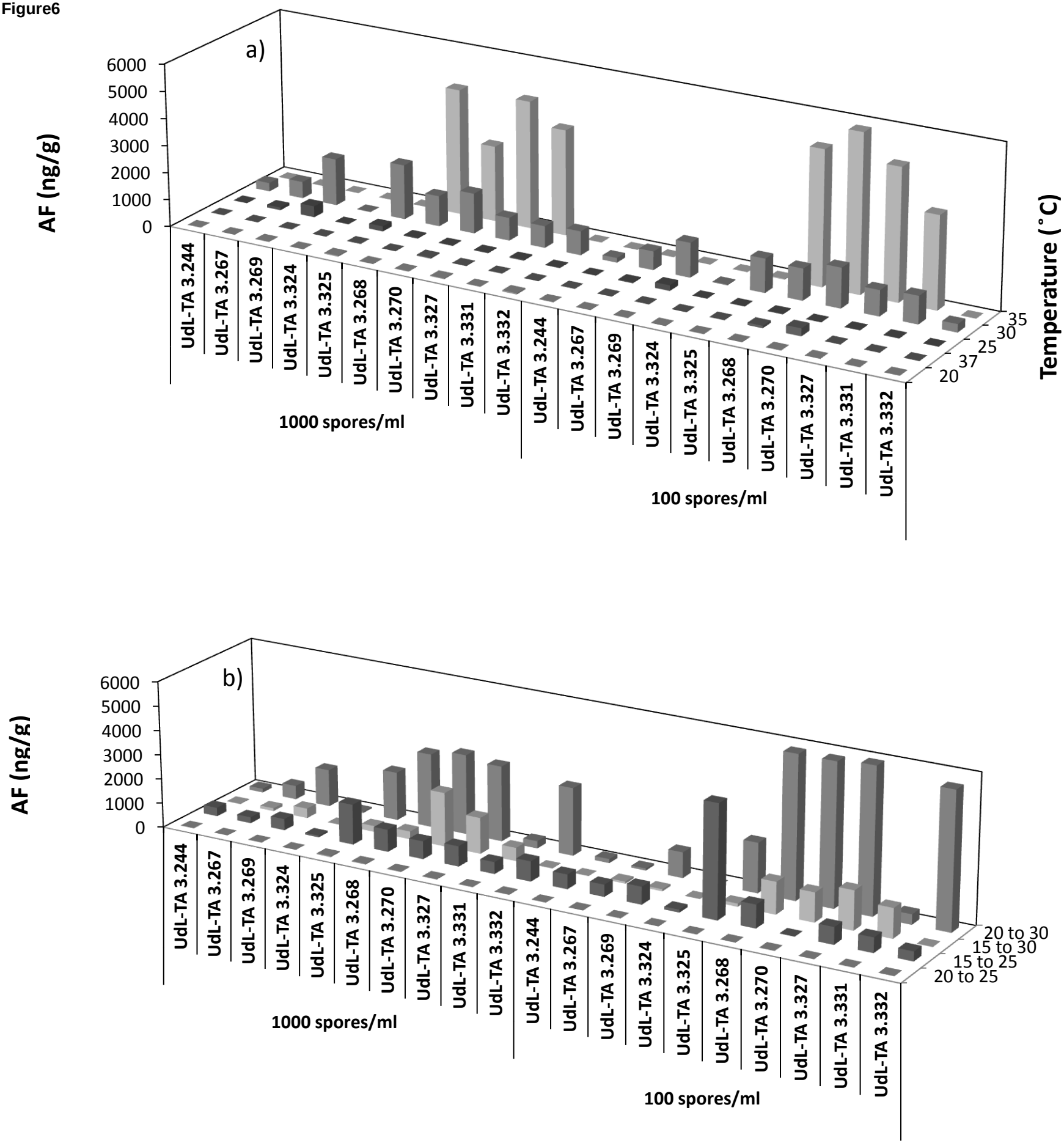

b)
Observed TTD (hours)
Predicted TTD (hours)
20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180 190 200

Figure6


a)
AF (ng/g)
6000
5000
4000
3000
2000
1000
0
UdL-TA 3.244
UdL-TA 3.267
UdL-TA 3.269
UdL-TA 3.324
UdL-TA 3.325
UdL-TA 3.268
UdL-TA 3.270
UdL-TA 3.327
UdL-TA 3.331
UdL-TA 3.332
1000 spores/ml
100 spores/ml
Temperature (˚C)
35
30
25
37
20
b)
AF (ng/g)
20 to 30
15 to 30
15 to 25
20 to 25
Temperature (˚C)