Year-to-year variation in the density of **Ixodes ricinus** ticks and the prevalence of the rodent-associated human pathogens **Borrelia afzelii** and **B. miyamotoi** in different forest types

Year-to-year variation in the density of *Ixodes ricinus* ticks and the prevalence of the rodent-associated human pathogens *Borrelia afzelii* and *B. miyamotoi* in different forest types.

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24   ABSTRACT

25   The human pathogens *Borrelia afzelii*, which causes Lyme borreliosis and *B. miyamotoi*, which causes

26   relapsing fever, both circulate between *Ixodes ricinus* ticks and rodents. The spatiotemporal dynamics in the

27   prevalence of these pathogens have not yet been fully elucidated, but probably depend on the spatiotemporal

28   population dynamics of small rodents. We aimed to evaluate the effect of different forest types on the density

29   of infected nymphs in different years and to obtain more knowledge about the spatial and temporal patterns

30   of ticks and tick-borne pathogens. We analysed unfed nymphal ticks from 22 stands of four different forest

31   types in Belgium in 2009, 2010, 2013 and 2014 and found that the density of nymphs in general and the

32   density of nymphs infected with *B. afzelii* and *B. miyamotoi* varied yearly, but without temporal variation in

33   the infection prevalence. The yearly variation in density of infected nymphs in our study thus seems to be

34   caused most by the variation in the density of nymphs, which makes it a good predictor of disease risk. The

35   risk for rodent-associated tick-borne diseases also varied between forest types. We stress the need to

36   elucidate the contribution of the host community composition to tick-borne disease risk.

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INTRODUCTION

Tick-borne diseases are a growing public health concern (Dantas-Torres et al., 2012). The most common tick-borne disease in the northern hemisphere is Lyme borreliosis, which is caused by some genospecies of the *Borrelia burgdorferi* sensu lato (s.l.) complex (Stanek et al., 2012), of which *B. afzelii* is the most prevalent in many Western European regions (Bingsohn et al., 2013; Gassner et al., 2011; Rauter and Hartung, 2005; Ruyts et al., 2016). Both *B. afzelii* and *B. miyamotoi*, the latter causing relapsing fever, circulate in the same tick species and the same vertebrate hosts (Cosson et al., 2014; Hanincová et al., 2003). In Europe, *Ixodes ricinus* is the main vector for *B. afzelii* transmission to humans (Piesman and Gern, 2004) and especially the host-seeking nymphs contribute most to the Lyme borreliosis risk (Barbour and Fish, 1993). In addition, this tick species is an important carrier for *B. miyamotoi*, and as with other tick-borne pathogens, *B. afzelii* regularly co-occurs with *B. miyamotoi* in the same tick individuals (Cosson et al., 2014; Gern et al., 2010; Kjelland et al., 2015).

The different genospecies of *B. burgdorferi* s.l. and *B. miyamotoi* each appear to be associated with a particular host species, or a range of hosts. *Borrelia afzelii* is commonly transmitted to ticks by small rodents, such as the wood mouse (*Apodemus sylvaticus* Linnaeus, 1758) and the bank vole (*Myodes glareolus* Schreber, 1780) (Hanincová et al., 2003; Humair et al., 1995). Also Eurasian red squirrels (*Sciurus vulgaris* Linnaeus, 1758) and European hedgehogs (*Erinaceus europaeus* Linnaeus, 1758) have been suggested to transmit *B. afzelii* to ticks (Jahfari et al., 2017; Pisanu et al., 2014; Ruyts et al., 2017; Skuballa et al., 2012). Like *B. afzelii*, *B. miyamotoi* appears to be associated with rodents (Barbour et al., 2009; Cosson et al., 2014; Taylor et al., 2013).

The preferential habitat of *I. ricinus* is forest, due to the sheltered microclimate and availability of vertebrate hosts for their blood meals (Gray et al., 1998; Lindstrom and Jaenson, 2003). Juvenile ticks (larvae and nymphs) generally feed most often on small to medium sized hosts, while adults tend to feed on medium sized to large hosts. A recent European meta-analysis including 44 hosts, however, showed that only a few host species (small rodents, thrushes and roe deer) feed the majority of *I. ricinus* individuals (Hofmeester et al., 2016). Roe deer are generally the most important feeding host for female ticks in Europe and are important in the maintenance and reproduction of *I. ricinus* populations (Gray, 1998; Hofmeester et al., 2016; Ruiz-Fons and Gilbert, 2010). In most regions, larvae mainly feed on small rodents, and rodents are

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69 generally responsible for the majority of *B. burgdorferi* s.l. infections in *I. ricinus* larvae (Hofmeester et al.,

70 2016). The densities of small rodents such as wood mouse and bank vole in our study region, but also of

71 other important host species such as roe deer, are positively correlated with the presence of a shrub layer and

72 are higher in deciduous forests than in coniferous forests (Tack, 2013; Tack et al., 2012a). Furthermore,

73 infection prevalence of nymphs with *B. afzelii* tends to be higher in pine than in oak forests, which suggests

74 that small rodents feed more larvae in pine than in oak forests, relative to other host species (Ruyts et al.,

75 2016). The densities of nymphs are also highest in structure rich deciduous forests (Gray et al., 1998; Ruyts

76 et al., 2016; Tack et al., 2012b). Besides the type of forest, also the availability of seeds influences the

77 occurrence and population dynamics of rodents, which is shown to affect the density of nymphs (Ostfeld et

78 al., 2006, 2001; Tack, 2013; van Duijvendijk, 2016). Therefore, it is expected that the spatial and temporal

79 differences in population dynamics of small mammals are important in explaining the density of infected

80 nymphs, which is a commonly used tick-borne disease risk measure (Ostfeld et al., 2006).

81 The temporal dynamics in the prevalence of many important tick-borne pathogens, such as the Lyme

82 borreliosis bacteria, remain largely unclear. In the light of the reported rise in incidence of tick-borne

83 diseases in recent years, the study of the ecology and the spatial and temporal patterns of ticks, hosts and

84 tick-borne pathogens is becoming increasingly important (Estrada-Peña et al., 2011; Gray et al., 2009;

85 Randolph, 2010). With our temporal survey, we provide data on the annual variability of the impact of forest

86 characteristics on the density of ticks and the infection prevalence of the rodent-associated pathogens *B.*

87 *afzelii* and *B. miyamotoi*.

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89 MATERIALS AND METHODS

90 **Study area**

91 This study was performed in two forest sites in the Campine region in northern Belgium; one in the

92 municipality Postel (51°17'26.35" N, 5°11'40.11" E), the other between the municipalities Herselt and

93 Averbode (51°02'42.91" N, 4°57'17.19" E). The climate is temperate with warm summers (Peel et al.,

94 2007). Forests in this region mainly consist of even-aged homogenous stands of Scots pine (*Pinus sylvestris*

95 L), and to a smaller extent Corsican pine [*P. nigra* Arnold subsp. *laricio* (Poiret). Maire] interspersed with

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more diverse, structure-rich deciduous forests composed of pedunculate oak (*Quercus robur* L.), Northern red oak (*Q. rubra* L.), common beech (*Fagus sylvatica* L.), silver birch (*Betula pendula* Roth.) and downy birch (*B. pubescens* Ehrh.). The forests in the Campine region are frequently visited for recreational purposes and Lyme borreliosis incidence in this region is relatively high compared to other regions in Belgium (Linard et al., 2007; Vanthomme et al., 2012).

Forest stand selection

The 22 forest stands we used in this study were selected in the framework of the study of Tack et al. (2012b) and were also studied in Ruyts et al. (2016). The forest stands lie in a larger matrix of forest stands of different forest types. We investigated stands of four different forest types, i.e. stands dominated either by pines ('pine stands') or oaks ('oak stands'), with (> 50% of the forest floor covered by shrubs) or without (< 25%) a well-developed shrub layer. The 22 studied stands included five pine stands without a shrub layer, six pine stands with a shrub layer, six oak stands without a shrub layer and five oak stands with a shrub layer. The forest stands were on average 1 ha in size, ranging from 0.5 to 4 ha. In our study region, the years 2006, 2007 and 2011 were mast years of pedunculate oak and 2011 was a mast year of beech (Nussbaumer et al., 2016). Corsican pine experienced a high seed crop in 2012 and 2013 and Scots pine in 2013 (Verstraeten A., personal communication). No data for these pine species are available for our region before 2009.

Data collection

Questing nymphs were sampled three to four times per year in a fixed representative part of each forest stand between June and October in 2009, 2010, 2013 and 2014. All stands were sampled with comparable intensity and in the same period each year. For the exact procedure of tick sampling we refer to Ruyts et al. (2016). The differences in structure and composition of the herbaceous community between the different stands were negligible so that the sampling could be performed in a standardized way (Tack et al., 2012a). The stands were sampled in a random order each time, to account for daily fluctuations in temperature and humidity during the sampling sessions. Nymphs were removed from the blanket after sampling each transect and transferred to vials containing 70% ethanol and afterwards stored at -22 °C. We counted and pooled nymphs from all sampling occasions from each year per forest stand. From each pool, 35 individual nymphs were randomly selected to examine for infection with *B. burgdorferi* s.l. genospecies and *B. miyamotoi*. For the procedure of DNA extraction of the individual nymphs and the simultaneous detection of *B. burgdorferi* s.l.

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124 and *B. miyamotoi* by multiplex qPCR, and for the identification of *B. burgdorferi* s.l. genospecies, we refer

125 to the methods described in Hansford et al. (2014). As the conventional *Borrelia*-PCR followed by Sanger

126 sequencing is less sensitive than our duplex *Borrelia*-qPCR, we could not assign a genospecies to all ticks

127 that were *B. burgdorferi* s.l.-positive by qPCR. To correct for this shortcoming, we approximated the

128 infection prevalence of nymphs with each *B. burgdorferi* s.l. genospecies for each plot following the

129 procedure described in Jahfari et al. (2017).

130 **Statistical analysis**

131 All analyses were conducted in R version 3.3.1 (R Core Team, 2017). DON is the average yearly density of

132 nymphs per plot. The nymphal infection prevalence (NIP) is the proportion of infected nymphs per year,

133 averaged over all sampling occasions per year per plot, and the density of infected nymphs (DIN) is the

134 product of DON and NIP. We calculated NIP and DIN for the *B. burgdorferi* s.l. complex, for each *B.*

135 *burgdorferi* s.l. genospecies and for *B. miyamotoi*. Due to low numbers for *B. burgdorferi* s.l. genospecies

136 other than *B. afzelii*, only NIP_{sl}, DIN_{sl}, $NIP_{afzelii}$, $DIN_{afzelii}$, $NIP_{miyamotoi}$, and $DIN_{miyamotoi}$ were included in the

137 statistical analyses.

138 We used linear mixed-effect models (*lme*) from the package *nlme* (Pinheiro et al., 2015) to explore the effect

139 of sampling year and forest characteristics on the response variables DON, NIP_{sl}, DIN_{sl}, $NIP_{afzelii}$, $DIN_{afzelii}$,

140 $NIP_{miyamotoi}$ and $DIN_{miyamotoi}$. As fixed effects, we used sampling year (levels '2009', '2010', '2013', '2014'),

141 the dominant tree species ('pine' or 'oak'), the presence of a shrub layer ('yes' or 'no') and all two-way

142 interactions. We added forest stand as a random effect to take into account the repeated measures in each

143 stand. Significance of the predictor variables in all model fits were assessed using analysis of variance

144 (ANOVA) with Chi-square (χ^2) test and we checked for heterogeneity of the residuals following the

145 approach described in Zuur *et al.* (2009). Finally, to estimate if changes in DON correlate to changes in NIP,

146 we performed a Spearman Rank Correlation using the package *Hmisc* (Harrell et al., 2016) on DON and

147 NIP_{sl}, $NIP_{afzelii}$ and $NIP_{miyamotoi}$.

148 We did not statistically test the effect of weather variables such as precipitation and temperature on the tick-

149 borne disease risk, since our sample size of four years and 22 stands was too low.

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151   RESULTS

152   In the 22 forest stands, a total of 21,376 questing *I. ricinus* nymphs were collected. We used 3,080 nymphs

153   for further analysis. Overall, 17.63% of the analysed nymphs was infected with at least one pathogen. We

154   identified six different *B. burgdorferi* s.l. genospecies in 341 of the 471 (72.4%) infected nymphs, namely *B.*

155   *afzelii*, *B. garinii*, *B. burgdorferi* s.s., *B. valaisiana*, *B. spielmanii* and *B. bavariensis* (Supplementary Table),

156   but we were unable to identify the genospecies in 130 *B. burgdorferi* s.l.-positive nymphs. Thirteen nymphs

157   were co-infected with *B. burgdorferi* s.l. and *B. miyamotoi*. For eight of these co-infected nymphs, *B.*

158   *miyamotoi* occurred together with *B. afzelii*. The *B. burgdorferi* s.l. genospecies in the remaining five cases

159   of co-infection could not be identified.

160   Figure 1 visualizes DON, $NIP_{afzelii}$ and $DIN_{afzelii}$ in each year. DON ($p < 0.01$) significantly differed among

161   years, with highest values in 2010 and lowest in 2014 (Table 1 and Fig. 1). DON was consistently higher in

162   oak forests than in pine forests (Fig. 1, Table 1). DON was significantly higher in stands with a shrub layer

163   than in stands without a shrub layer in 2009 and 2010, but no difference was found in 2013 and 2014.

164   The variables NIP_{sl}, $NIP_{afzelii}$ and $NIP_{miyamotoi}$ did not show significant temporal variation (Table 1). $NIP_{afzelii}$

165   was significantly higher in pine forests, consistently throughout the years (Table 1 and Fig. 1). We found no

166   correlation between DON and NIP_{sl} ($p = 0.17$, $\rho = -0.15$), between DON and $NIP_{afzelii}$ ($p = 0.24$, $\rho = -0.13$) or

167   between DON and $NIP_{miyamotoi}$ ($p = 0.32$, $\rho = -0.11$).

168   Like DON, DIN_{sl} ($p = 0.02$) significantly differed among years, with highest values in 2010 and lowest in

169   2014 (Table 1 and Fig. 1). $DIN_{miyamotoi}$ and $DIN_{afzelii}$ did not show significant temporal variation (Table 1).

170   Like DON, DIN_{sl} and $DIN_{miyamotoi}$ were higher in oak forests than in pine forests, consistently throughout the

171   years (Table 1). DIN_{sl} was significantly higher in stands with a shrub layer compared to stands without a

172   shrub layer in 2010, while in the other years, no significant effect could be detected of the presence of a

173   shrub layer.

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175   DISCUSSION

176   In this temporal survey, we looked at the inter-annual dynamics in tick densities and the infection prevalence

177   of tick-borne bacteria, with special attention to the rodent-associated human pathogens *B. afzelii* and *B.*

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178 *miyamotoi*, in relation to forest types in Belgium. Our results indicate that the risk of rodent-associated tick-

179 borne disease varies both between different types of forest and between years. This spatiotemporal variation

180 can be related to the response of both ticks and hosts to the biotic and abiotic conditions influenced by the

181 dominant tree species, and can be predicted by the density of nymphs.

182 In our study, the rodent-associated pathogens *B. afzelii* and *B. miyamotoi* were the most common bacteria in

183 the investigated nymphs. The bird-associated *B. burgdorferi* s.l. genospecies *B. garinii* and *B. valaisiana*

184 occurred at low infection prevalence in our study sites. Together, this suggests that rodents are most likely

185 the most important feeding hosts for larvae in our study area, as stated by Hofmeester et al. (2016). In our

186 study, *B. miyamotoi* displayed co-infection with *B. afzelii*, which supports the assumption that they share the

187 same hosts (Barbour et al., 2009; Cosson et al., 2014; Taylor et al., 2013).

188 Our results show that DON, but not NIP, displays inter-annual fluctuations. Some European studies have

189 reported that an increased supply of acorns can increase the population density of wood mouse and bank vole

190 the next year (Tack, 2013; van Duijvendijk, 2016). Moreover, they show that this increased rodent density

191 leads to more feeding opportunities for larvae, and a high DON one year later, while NIP remains stable.

192 Also densities of other host species, such as roe deer, red squirrel and wild boar, may increase after a high

193 seed crop of oak, beech or pine (Cutini et al., 2013; Tixier and Duncan, 1996; Wauters et al., 2004; Wauters

194 and Lens, 1995). Oak experienced a high seed crop in 2006, 2007 and 2011, beech in 2011 and pine in 2012

195 and 2013. Based on this, we would expect DON to be highest in the years 2009, 2013 and 2014. However,

196 DON is highest in 2010 and 2013. Yearly variation in weather conditions such as temperature and the

197 amount of precipitation can also influence DON. Since ticks are sensitive to desiccation (Needham and Teel,

198 1991), they will be more prone to death in dry conditions, or will seek shelter in the litter layer or lower

199 vegetation which makes it more difficult to collect them with the standard sampling methods and thereby

200 biasing the results. In our study, it is not possible to conclude if mast years or weather conditions affect

201 DON, as these and other possible influencing factors are not accounted for.

202 Lyme borreliosis incidence has increased significantly the last decades in many European countries

203 (Ducoffre, 2010; Hofhuis et al., 2006; Sprong et al., 2012). We found no clear pattern in DON, NIP_{sl} or DIN_{sl}

204 but rather DON and DIN_{sl} varied from one year to the other. Similar to our results, Estrada-Peña et al. (2011)

205 detected no specific temporal trend at the European level in the prevalence of *B. burgdorferi* s.l. genospecies

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206 and relate the prevalence of genospecies across Europe to temperature and vegetation stress, which are

207 important drivers of both tick and host populations. Like in other studies (James et al., 2013; Jouda et al.,

208 2004; Vourc'h et al., 2016), but contrary to Tälleklint and Jaenson (1996), we found no correlation between

209 DON and NIP. As NIP in our study did not vary from year to year, the temporal variation in DIN resembles

210 the temporal variation in DON. This confirms that DON can be a good predictor of disease risk, as already

211 suggested by e.g. Jaenson et al. (2009). The relationship between DON and NIP, however, can depend on the

212 specific host community composition (Kurtenbach et al., 2006; Tälleklint and Jaenson, 1996; van Buskirk

213 and Ostfeld, 1995).

214 In accordance with other studies (Ruyts et al., 2016; Tack et al., 2012b), we found a higher DON in oak

215 forests and a higher $NIP_{afzelii}$ in pine forests. The higher DON in oak stands can be explained by the more

216 favourable biotic and abiotic conditions for ticks in oak forests than in pine forests, such as a better

217 microclimate or a higher abundance of hosts (Gray et al., 1998). Previous research has shown that oak forests

218 in our study region harbour higher densities of small rodents and roe deer compared to pine forests, and thus

219 more feeding opportunities for ticks (Tack, 2013; Tack et al., 2012a). Although the densities of small rodents

220 are higher in oak than in pine forests, it is possible that, due to their wide ecological tolerance (Douglass et

221 al., 1992), wood mouse and bank vole contribute more to the host community in pine than in oak forests,

222 relative to other host species. This way they feed relatively more larvae in pine forests, as already suggested

223 by Ruyts et al. (2016). Squirrels are also generally more abundant in pine than in oak forests (Wauters and

224 Lens, 1995). Since squirrels are, like mice and voles, believed to be associated with *B. afzelii* (Hanincová et

225 al., 2003; Humair et al., 1995; Pisanu et al., 2014; Ruyts et al., 2017), this might explain the higher $NIP_{afzelii}$

226 in pine than in oak stands.

227 From our results, we may conclude that the density of nymphs can be used to predict yearly variation in tick-

228 borne disease risk. We found that the effect of the dominant tree species on the density of nymphs, which

229 reflects changes in biotic and abiotic conditions, is consistent through time. In this study, we did not directly

230 examine the host community of the ticks. Further research should therefore try to determine the exact

231 contribution of the different host species and of the whole host community to the enzootic cycle of human

232 pathogens, and to test the effect of weather conditions and different host community compositions to the

233 tick-borne disease risk.

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234   CONFLICT OF INTEREST

235   The authors declare no conflict of interest.

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Fig. 1. The density of nymphs (DON), nymphal infection prevalence of the rodent associated pathogen *Borrela afzelii* ($NIP_{afzelii}$) and density of nymphs infected with *B. afzelii* ($DIN_{afzelii}$) in the different sampling years averaged over pine and oak stands (mean ± S.D.).

Table 1. The effect of sampling year, dominant tree species and presence of a shrub layer and their two-way interactions on density of nymphs (DON), nymphal infection prevalence of *Borrelia burgdorferi* s.l. (NIP_{sl}), *B. afzelii* ($NIP_{afzelii}$) and *B. miyamotoi* ($NIP_{miyamotoi}$), and density of nymphs infected with *B. burgdorferi* s.l. (DIN_{sl}), *B. afzelii* ($DIN_{afzelii}$) and *B. miyamotoi* ($DIN_{miyamotoi}$). Values represent F-values obtained by ANOVA (* $p < 0.05$). Higher F-values indicate higher variation in the response variable.

	year	tree	shrub	tree:shrub	tree:year	shrub:year
DON	13.27*	20.29*	8.32*	0.2	2.02	5.90*
NIP_{sl}	0.16	4.17	<0.01	0.38	0.03	0.65
DIN_{sl}	3.82*	6.54*	6.36*	1.04	0.29	2.49
$NIP_{afzelii}$	0.63	9.43*	0.9	0.5	1.42	0.18
$DIN_{afzelii}$	1.65	1.59	1.49	1.12	0.19	0.7
$NIP_{miyamotoi}$	0.27	0.06	<0.01	0.71	0.6	1
$DIN_{miyamotoi}$	2.38*	6.85*	0.2	0.5	0.36	1.56

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423 **Supplementary table 1.** The infection prevalence (%) of *Ixodes ricinus* nymphs with *Borrelia miyamotoi* or

424 a distinct *B. burgdorferi* s.l. genospecies in 2009, 2010, 2013 and 2014 in each studied forest type, averaged

425 over all forest stands from that forest type. We approximated the nymphal infection prevalence of the *B.*

426 *burgdorferi* s.l. genospecies to correct for the samples that were positive in RT-PCR but could not be

427 identified to genospecies level, as written in the text.

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Bacteria	Year	Pine		Oak	
		without shrub	with shrub	without shrub	with shrub
B. afzelii	2009	17.9	9.1	9.0	11.1
	2010	10.6	10.0	9.1	8.8
	2013	16.0	16.0	9.6	6.3
	2014	15.3	13.5	6.3	6.0
B. garinii	2009	0	1.3	4.8	2.0
	2010	0	3.5	0.5	0.6
	2013	0	1.0	1.5	2.9
	2014	0	1.8	0.6	7.4
B. burgdorferi **s.s.**	2009	3.3	3.6	0	1.7
	2010	5.4	1.8	1.2	4.3
	2013	0	0.6	4.0	0.6
	2014	1.2	0.9	0	0
B. valaisiana	2009	0	0.8	0.6	0
	2010	0	1.4	0.6	0
	2013	0	0	0.6	0.6
	2014	0	1.0	1.7	0
B. spielmanii	2009	0	0	0	0
	2010	0	0	0	0
	2013	0	0	0.5	1.1
	2014	0.7	0.0	1.3	1.5
B. bavariensis	2009	0	0	0	0
	2010	0	0	0	0.6
	2013	0	0	0	0
	2014	0	0	0	0
B. miyamotoi	2009	0.6	3.8	3.8	2.3
	2010	2.9	1.9	3.3	3.4
	2013	3.4	2.9	3.8	1.7
	2014	1.7	3.3	1.9	2.3

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