Supplementary material

Exposure of yellow-legged gulls to *Toxoplasma gondii* along the Western Mediterranean coasts: tales from a sentinel

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Appendix A. Supplementary text and figures

S1. Study population

Figure S1.1. Surroundings of a yellow-legged gull nest on Corrège (France). Note the presence of probable marine resources such as seashells (left) and probable refuse tips such as large animal bones (e.g., bottom-right) and olive pits, illustrating the opportunistic foraging behaviour of this species. Picture: Amandine Gamble.

Table S1.1. Prevalences of anti-*T. gondii* antibodies in yellow-legged gull egg yolk samples measured by ELISA. c.i. = confidence interval (here Clopper-Pearson confidence interval).

Site	Coordinates (latitude, longitude)	Year	Sample size (nests)	Prevalence [95% c.i.]
Riou	43.1779, 5.3783	2009	30	0.17 [0.06; 0.35]
		2106	31	0.35 [0.19; 0.55]
Frioul	43.2647, 5.2893	2009	30	0.20 [0.08; 0.39]
		2010	33	0.21 [0.09; 0.39]
		2011	30	0.13 [0.04; 0.31]
		2012	30	0.17 [0.06; 0.35]
		2014	16	0.06 [0.00; 0.30]
		2016	29	0.45 [0.26; 0.64]
Carteau	43.3779, 4.8579	2009	29	0.14 [0.04; 0.32]
		2016	29	0.19 [0.16; 0.39]
Vic-la-Gardiole	43.4921, 3.8476	2016	27	0.52 [0.32; 0.71]
Gruissan	43.1099, 3.1067	2009	30	0.33 [0.17; 0.53]
		2010	30	0.37 [0.20; 0.56]
		2012	30	0.23 [0.10; 0.42]
		2014	32	0.41 [0.24; 0.59]
		2016	31	0.32 [0.17; 0.51]
Hortel	42.9057, 3.0062	2009	30	0.23 [0.10; 0.42]
Sidrière	42.8998, 3.0099	2009	30	0.30 [0.15; 0.49]
		2014	12	0.08 [0.00; 0.38]
		2016	29	0.28 [0.13; 0.47]
Corrège	42.8546, 3.0225	2009	30	0.30 [0.15; 0.49]
		2016	30	0.50 [0.31; 0.69]
Medes	42.0476, 3.2219	2009	29	0.10 [0.02; 0.27]
		2010	30	0.13 [0.04; 0.31]
		2011	30	0.10 [0.02; 0.27]
		2012	30	0.07 [0.01; 0.22]
		2013	23	0.04 [0.00; 0.22]
		2015	32	0.22 [0.09; 0.40]
		2016	30	0.17 [0.06; 0.35]
Barcelona	41.3875, 2.1879	2016	32	0.12 [0.04; 0.29]
Ebro Delta	40.5536, 0.6198	2016	22	0.50 [0.22; 0.72]
Dragonera	39.5853, 2.3202	2016	8	0.50 [0.16; 0.83]
Illa de l'Aire	39.8012, 4.2904	2016	30	0.33 [0.17; 0.53]
Sfax	34.7078, 10.7506	2009	30	0.03 [0.00; 0.17]
Djerba	33.6528, 10.9831	2009	37	0.11 [0.03; 0.25]

Table S1.2. Isotopic ratios of carbon ($^{13}C/^{12}C$; $\delta^{13}C$), nitrogen ($^{15}N/^{14}N$; $\delta^{15}N$) and sulphur ($^{34}S/^{32}S$; $\delta^{34}S$) measured in yellow-legged gull albumen samples. c.i. = confidence interval.

Site	Year	Sample size (nests)	Mean $\delta^{13}C$ [95% c.i.]	Mean $\delta^{15}N$ [95% c.i.]	Mean $\delta^{34}S$ [95% c.i.]
Frioul	2010	10	-21.66 [-22.04; -21.28]	10.53 [10.03; 11.03]	9.97 [8.75; 11.19]
Gruissan	2016	10	-22.23 [-22.61; -21.85]	9.89 [9.28; 10.5]	7.85 [7.37; 8.33]
Sidrière	2014	7	-21.66 [-22.08; -21.24]	9.01 [8.50; 9.52]	8.09 [6.89; 9.29]
	2016	15	-22.42 [-22.68; -22.16]	8.93 [8.65; 9.21]	8.02 [7.1; 8.94]
Medes	2010	13	-21.13 [-21.59; -20.67]	10.60 [10.16; 11.04]	7.15 [6.17; 8.13]
	2011	12	-21.1 [-21.45; -20.75]	10.48 [10.04; 10.92]	6.72 [5.51; 7.93]
	2012	8	-20.24 [-20.95; -19.53]	10.35 [8.60; 12.1]	6.95 [5.98; 7.92]
	2015	15	-20.38 [-20.83; -19.93]	10.79 [10.4; 11.18]	6.61 [5.92; 7.3]
	2016	15	-20.10 [-20.38; -19.82]	10.87 [10.36; 11.38]	7.59 [6.62; 8.56]
Barcelona	2016	10	-20.93 [-21.54; -20.32]	9.95 [9.45; 10.45]	9.03 [7.04; 11.02]
Ebro Delta	2016	14	-21.11 [-21.56; -20.66]	11.41 [10.7; 12.12]	13.63 [11.97; 15.29]

S2. Evaluation of the immunoassays

This study is the first to report the detection of anti-*Toxoplasma gondii* antibodies in gull egg yolk samples, and one of the few to report their detection in seabird in general (Cabezón et al., 2016; Dubey, 2002 for a review). Notably, the ID Screen® enzyme-linked immunosorbent assay (ELISA) had never been used on gull samples before but its availability and ease of use make it appealing. We thus included several validation procedures in our laboratory analyses to strengthen the robustness of the results we report. Overall, the ELISA was proven repeatable at a much higher level than the MAT at the sample level (*i.e.*, repeated analyses conducted on a given sample) but note that the MAT had never been used on egg yolk samples before to our knowledge. At the nest level (*i.e.*, repeated sampling of eggs in a given nest), the detection probability was suitable. In addition, the ELISA and the MAT lead to the same prevalences of anti-*T.gondii* antibodies at the colony scale. These results are in line with previous studies suggesting that ELISA are generally suitable to detect anti-*T. gondii* antibodies in domestic and wild animals (e.g., Sharma et al., 2018; Gamble et al., 2005).

Details of the ELISA procedure

The ELISA was carried out following the "sensitive" protocol suggested by the manufacturer, *i.e.*, 50 µl of diluted yolk samples were mixed with 50 µl of dilution buffer in each well. Outcomes are expressed as the optical density (OD) read at 450nm of the resulting solution after correction for inter-plate variations (see Lobato et al., 2011).

Repeatability of the ELISA at the sample level

Prior to the ELISA, two diluted samples were prepared from each yolk on a subset of 40 yolk samples selected on a range of ODs, all collected in Gruissan in 2009 or 2010. The dilution step was part of the ELISA procedure and carried out according to the manufacturer instructions (*i.e.*, yolk samples were diluted in the IDvet Wash Buffer composed of 0.05% Tween 20 diluted in phosphate buffered saline). The repeatability was assessed by a major axis regression using the '*lmodel2*' R package (Legendre, 2013) with the OD of dilution 1 as the explicative variable (x) and the OD of dilution 2 as the response variable (y). The order of the dilutions (dilution 1 *versus* dilution 2) was randomized.

The ELISA thus proved to be repeatable on a subset of samples ($r^2 = 0.99$, y = 0.00 [-0.01; 0.02] + 0.99 [0.95; 1.03] × x, with 95% confidence interval between brackets, n = 40; Figure S2.1).

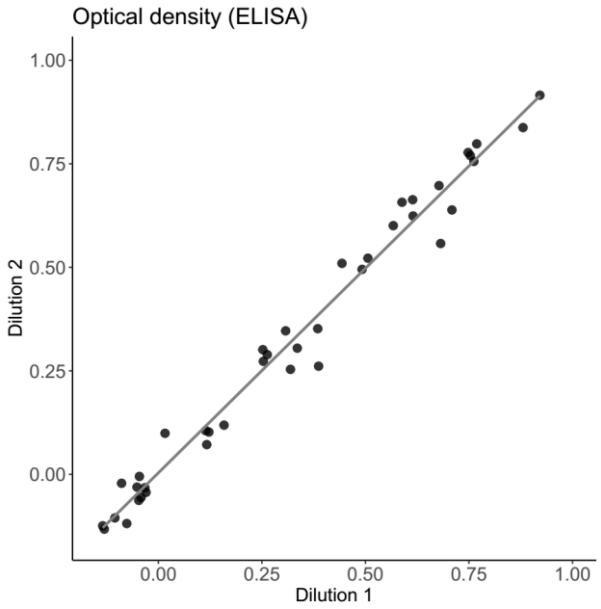

Figure S2.1. Evaluation of the repeatability of the dilution step: correlation between the Optical Densities (ODs) of two dilutions originating from the same yolk sample measured using an ELISA targeting anti-*Toxoplama gondii* antibodies. The order of dilutions (dilution 1 *versus* dilution 2) is randomized on the graphic.

ELISA positivity threshold

The ELISA we used was originally designed to detect chicken IgY. The use of anti-chicken IgY to detect the IgY of various seabird species has previously been validated using an experimental design based on vaccination (Garnier et al., 2017). However, because anti-chicken IgY (used to detected chicken IgY) may not have the same affinity for gull antibodies than for chicken antibodies, the positivity threshold of the test had to be adapted to the studied species. For this, we used the approach described on Garnier et al. (2017) and fitted a mixture of normal distributions to the values of ODs to distinguish a group of seropositive and a group of seronegative individuals. The positivity threshold was thus fixed as the mean + 2 standard deviations of the normal distribution followed by the ODs of the negative samples.

The ELISA positivity threshold was estimated at an OD value of 0.41 (Figure S2.2).

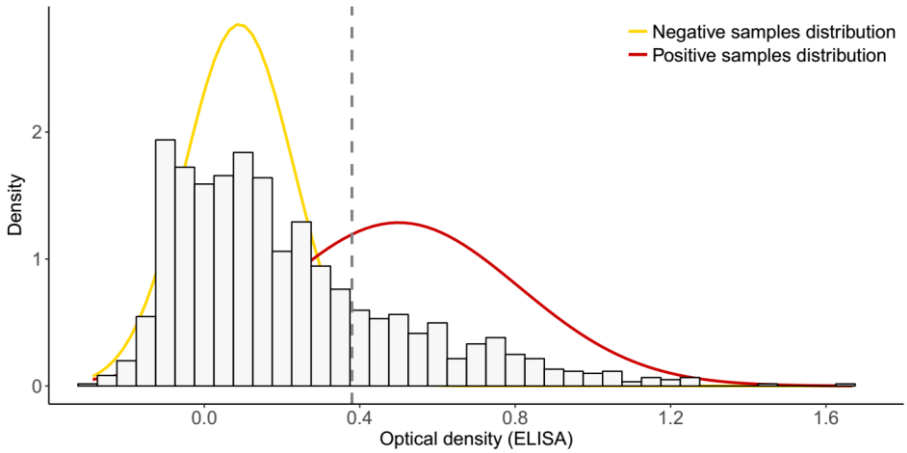

Figure S2.2. Distribution of optical densities resulting from an ELISA targeting anti-*T. gondii* antibodies in yellow-legged gull egg yolk samples. The histograms present the normalized counts of individuals and the curves correspond to the probability density function of the two normal distributions (negative and positive samples). The threshold value (dashed line) was estimated as the mean + 2 standard deviations of the normal distribution followed by the ODs of the negative samples.

Detection probability of the ELISA at the nest level

In order to assess if the detection of antibodies from an egg is a good proxy of the nest status, we used a patch occupancy approach (MacKenzie et al., 2002). This approach allows the estimation of the occupancy probability (*i.e.*, proportion of sites occupied by a species among searched sites) while accounting for the fact that detection probability of the targeted species is < 1. Similarly, when looking for antibodies (instead of species) in nests (instead of sites) by sampling eggs in these nests (instead of visiting sites), the detection probability may be < 1 in relation to the immunoassay sensitivity or differences among eggs of a same clutch. The patch occupancy approach has already been used to estimate the detection probability (corresponding to detect at least an egg as positive in a positive clutch) and occupancy probability (corresponding to the proportion of sites occupied by at least one individual of the targeted species, or here, to the proportion of nests in which at least one egg contains antibodies) of anti-avian influenza virus antibodies in the same samples in a previous study (Hammouda et al., 2014, 2011). This study reported a detection probability > 0.90, suggesting that the detection of antibodies from an egg is a good proxy of the nest status.

We thus fitted an occupancy model on the antibody data from the two colonies of Tunisia in which three eggs per clutch had been sampled. The dataset thus consisted of three observation occasions per site (corresponding to the three eggs collected per nest) in 67 sites (37 nests from

Djerba and 30 from Sfax). Two models were run: one with constant detection probability and occupancy probability [p(.) ψ(.)], and one with constant detection probability but colony-dependant occupancy probability [p(.) ψ(colony)]. The best model was selected based on Akaike Information Critera (AIC; Burnham and Anderson, 2002).These models were run using the '*unmarked*' R package (Fiske and Chandler, 2011). Non-transformed estimates are reported with their standard error (s.e.).

The selected model included an association between the occupancy probability and the colony [p(.) ψ(colony)]. The estimated detection probability was at 0.69 with a large standard error (non-transformed estimate ± s.e. = 0.81 ± 0.52, p = 0.12). The estimated prevalences of antibodies (corresponding to the occupancy probability) were at:

- 0.17 (non-transformed estimate ± s.e. = -1.61 ± 0.45, $p < 0.01$) at Djerba,
- 0.15 (non-transformed estimate ± s.e. = -1.73 ± 1.11, p = 0.12) at Sfax.

The large standard error of the estimate of the detection probability is probably due to the low prevalences of antibodies in the considered colonies (only 7/67 nests with at least one egg detected as antibody-positive and estimated prevalences of antibodies ≤ 0.17). Note that in the case of avian influenza viruses, the estimated prevalences of antibodies varied between 0.35 and 0.89 (Hammouda et al., 2014, 2011). In the case of *T. gondii*, because the prevalence of antibodies in the considered colonies is low, ideally the design should include more sampling occasions per site (*i.e.*, more eggs per nest; Mackenzie and Royle, 2005) but it is of course not possible because gulls only lay three eggs in natural conditions.

Concordance between the ELISA and the MAT

No tests is consider as gold standard for the detection of anti-*T. gondii* antibodies in wild species because of the difficulty to measure specificity and sensitivity in non-model species. In the absence of a true gold standard, we chose to compare the results of the ELISA to the most commonly used assay for *T. gondii* serosurveys in the wild: the Modified Agglutination Test (MAT). The MAT has notably previously been used in wild birds (e.g., Cabezón et al., 2011; Sandström et al., 2013), but never on egg yolk samples to our knowledge. Although both assays are generally used to track the same event, *i.e.*, exposure to *T. gondii*, they rely on different molecular interactions. More specifically, the ELISA targets IgY immunoglobulins binding anti-chicken IgY and the P30 antigen of *T. gondii* while the MAT targets IgY immunoglobulins that agglutinate the whole *T. gondii* parasite (Dubey and Desmonts, 1987). The two assays are

thus expected to give slightly different results, as observed in previous studies (Elmore et al., 2016; e.g., Shaapan et al., 2008; Zhu et al., 2012).

To compare ELISA and MAT results on gull egg yolk samples, proteins were extracted from 40 samples selected on a range of ODs (see above) using chloroform as previously described in (Gasparini et al. 2001). The MAT was conducted following the procedure described in (Dubey & Desmonts 1987) twice on each extract: once on the extract diluted at 1:3 and once on the pure extract. The positivity threshold was set at a titre of 6, samples with a non-null titre inferior to 6 being considered as doubtful and then treated as negative in order to be conservative in relation to gull exposure to *T. gondii*.

Agreement between qualitative outcomes (i.e., negative or positive) of the ELISA and the MAT was assessed by calculating Cohen kappa coefficient (κ) with the '*psych*' R package (Revelle, 2014). A $\kappa \leq 0.4$ indicates a poor agreement, $0.4 < \kappa \leq 0.8$ indicates a fair to good agreement, and $\kappa > 0.8$ indicates an excellent agreement (Fleiss, 1981). Correlation between their quantitative outcomes was assessed by calculating Spearman rank correlation coefficient (ρ) using the '*Hmisc*' package (Harrell Jr, 2013). The repeatability of the MAT was measured using the same statistics to compare the outputs of the analyses run on pure and diluted extracts.

Overall, the prevalence resulting from the two MAT measures and the ELISA were similar (Table S3). In more detail, agreement between the qualitative results of the two MAT measures was fair, similarly to the agreement between the results of the ELISA and MAT (Table S4). Quantitative results were positively correlated (Figure S2.3).

Table S2.1. Prevalences of anti-*T. gondii* antibodies in yellow-legged gull egg yolk resulting from the ELISA and the MAT run on the same samples. c.i. = confidence interval (here Clopper-Pearson confidence interval); n = sample size.

			Prevalence [95% c.i.]		
Site	Year	n	MAT on 1:3 extracts	MAT on pure extracts	ELISA
Gruissan	2009	45	0.87 [0.73; 0.95]	0.91 [0.79; 0.98]	0.84 [0.71; 0.94]
	2010	23	0.30 [0.13; 0.53]	0.26 [0.10; 0.48]	0.48 [0.27; 0.69]

Table S2.2. Agreement between qualitative outcomes (i.e., negative or positive) of the ELISA and the MAT targeting anti-*T. gondii* antibodies in yellow-legged gull egg yolk. κ = Cohen kappa coefficient; c.i. = confidence interval; n = sample size.

Assays	κ [95% c.i.]	p-value	n
MAT on 1:3 extracts *vs.* MAT on pure extracts	0.76 [0.60; 0.92]	< 0.01	40
ELISA *vs.* MAT on 1:3 extracts	0.50 [0.28; 0.71]	< 0.01	40
ELISA *vs.* MAT on pure extracts	0.62 [0.43; 0.81]	< 0.01	40

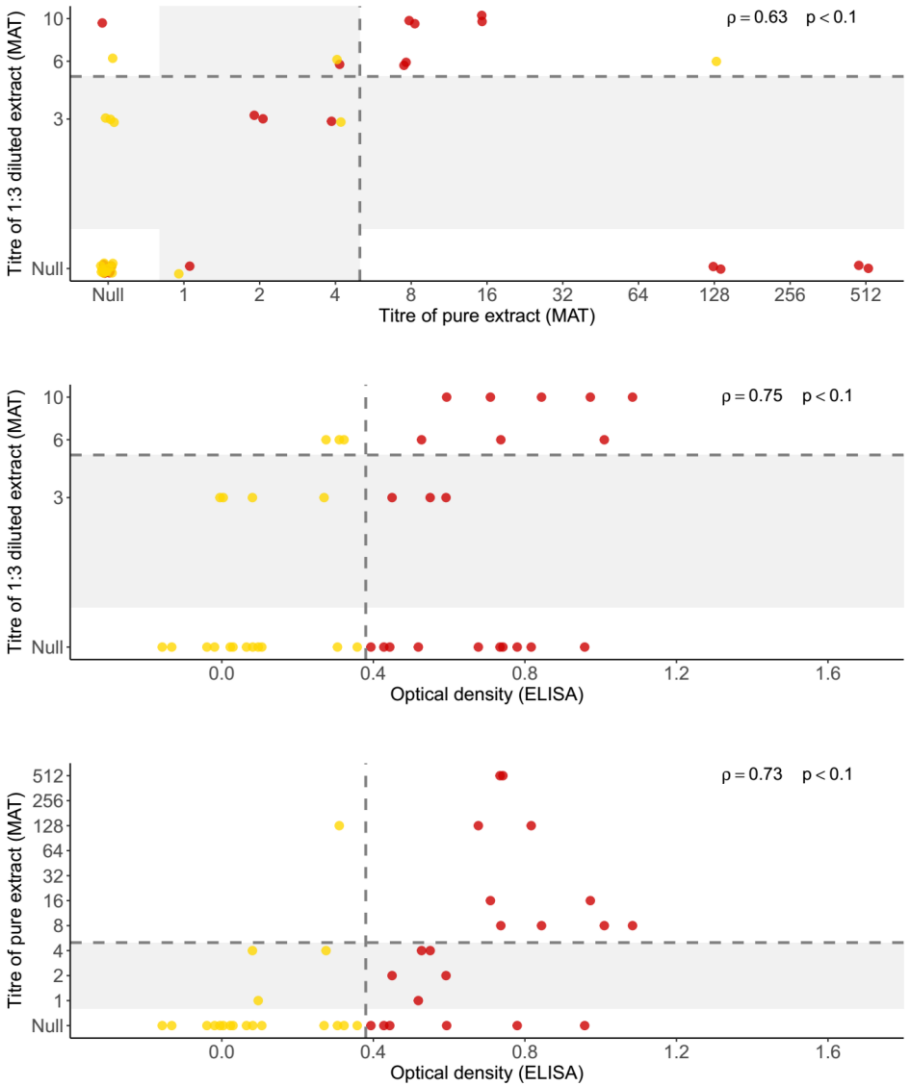

Figure S2.3. Correlation between *T. gondii* specific antibody levels in yellow-legged gull egg yolk samples using the ELISA and the MAT. Positivity threshold values are represented by dashed lines (samples left to or below the line are considered negative; samples right to or above the line are considered positive). Samples in the shaded area are doubtful and were considered as negative for the analyses. ρ = Spearman rank correlation coefficient.

S3. Selection of models explaining the variations of the prevalence of anti-*T. gondii* antibodies

Table S3.1. Exploration of the spatio-temporal variations of the prevalence of anti-*T. gondii* antibodies. The status of the nest (antibody-negative or –positive) was included as a binary variable. The site and the campaign were included as categorical variables. n = 414 samples. "+" denotes an additive effect, and "*" an interaction. AIC: Akaike information criterion. SE: standard error. The models with the lowest AIC (with a threshold of ΔAIC = 2) were selected and is indicated in bold. The likelihood ratio χ^2 of the considered variables are reported for the selected model(s).

Fixed effects	Degrees of freedom	AIC	Likelihood ratio test		
			Campaign	Site	Campain * Site
.	1	731			
Campaign	2	713			
Site	15	698			
Campaign + Site	**16**	**694**	$\chi^2 = 6$; $p = 0.02$	$\chi^2 = 47$; $p < 0.01$	-
Campaign * Site	22	702			

Table S3.2. Exploration of the temporal variations of the prevalence of anti-*T. gondii* antibodies. The status of the nest (antibody-negative or –positive) was included as a binary variable. The site was included as categorical variable, and the campaign as a continuous variable. n = 525 samples. "+" denotes an additive effect, and "*" an interaction. AIC: Akaike information criterion. The models with the lowest AIC (with a threshold of ΔAIC = 2) were selected and is indicated in bold. The likelihood ratio χ^2 of the considered variables are reported for the selected model(s).

Fixed effects	Degrees of freedom	AIC	Likelihood ratio test		
			Campaign	Site	Campain * Site
.	1	546			
Campaign	2	545			
Site	**3**	**527**	-	$\chi^2 = 23$; $p < 0.01$	-
Campaign + Site	**4**	**526**	$\chi^2 = 3$; $p = 0.07$	$\chi^2 = 23$; $p < 0.01$	-
Campaign * Site	**6**	**527**	$\chi^2 = 3$; $p = 0.07$	$\chi^2 = 23$; $p < 0.01$	$\chi^2 = 2$; $p = 0.31$

Table S3.3. Exploration of the relationship between the prevalence of anti-*T. gondii* antibodies and isotopic signatures. The status of the nest (antibody-negative or –positive) was included as a binary variable. The isotopic ratios of carbon, nitrogen and sulphur ($\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ respectively) were included as continuous variables. The site and the campaign were included as random effects. n = 129 samples. "+" denotes an additive effect. AIC: Akaike information criterion. The model with the lowest AIC (with a threshold of $\Delta AIC = 2$) was selected and is indicated in bold. The effect sizes of the considered variables are reported for the selected model(s).

Fixed effects	Degrees of freedom	AIC	Effect size ± standard error		
			$\delta^{13}C$	$\delta^{15}N$	$\delta^{34}S$
.	**3**	**153**	-	-	-
$\delta^{13}C$	**4**	**155**	0.19 ± 0.28	-	-
$\delta^{15}N$	**4**	**155**	-	0.14 ± 0.09	-
$\delta^{34}S$	**4**	**154**	-	-	0.09 ± 0.20
$\delta^{13}C + \delta^{15}N$	5	157			
$\delta^{15}N + \delta^{34}S$	5	156			
$\delta^{13}C + \delta^{34}S$	5	156			
$\delta^{13}C + \delta^{15}N + \delta^{34}S$	6	158			

S4. Intra-colony spatial structuration

Table S4.1. Quantification of the spatial-autocorrelation at the intra-colony scale. Geary C was calculated based on the nest status (antibody-negative or antibody-positive) and GPS locations within each colony. # positive = number of nests with an egg tested positive for anti-*T. gondii* antibodies; n = sample size.

Year	Site	Geary C	p-value	# positive / n
2016	Riou	0.75	0.14	11/31
	Frioul	0.88	0.39	13/29
	Vic-la-Gardiole	0.93	0.30	14/27
	Gruissan	1.11	0.67	8/27
	Corrège	0.77	0.15	15/30

S5. Isolation and genotyping of the *T. gondii* strain

Additional methods

Plasma samples of necropsied gulls were tested for anti-*T. gondii* antibodies before attempting to isolate the parasite from the brain and heart samples. Mice inoculated for parasite isolation were also tested for anti-*T. gondii* antibodies to assess the success of the inoculation step. For detection of anti-*T. gondii* antibodies in these cases, the MAT was preferred to the ELISA because of practical constraints. Raw OD values obtained by ELISA have indeed to be corrected for inter-plate variations, which requires to analyse replicated samples (that have also been analysed on the other plates used for the study). It is thus not relevant to run an ELISA only for a few samples. Additionally, this would lead to the loss of the non-used reagents. The three plasma samples of the necropsied gulls were not included in the quantitative analyses conducted on the egg yolk samples.

Additional results

Table S5.1. Multilocus microsatellite (MS) genotyping of the *T. gondii* strain obtained from a gulls originating from Ebro Delta (Spain) compared to 9 reference strains from the three main *Toxoplasma* lineages. BRC: Biological Resource Centre.

Isolate id (*Toxoplasma* BRC code)	MS genotype[b]															Genotypes	References
	TUB2	***W35***	***TgM-A***	***B18***	***B17***	***M33***	***MIV.1***	***MXI.1***	***M48***	***M102***	***N60***	***N82***	***AA***	***N61***	***N83***		
Gull strain																	
170329Lmic01ES (TgA 21067)	289	242	207	158	336	169	274	356	231	174	142	111	267	89	312	*Type II*	This study
Reference strains																	
GT1 (TgA 00004)	291	248	209	160	342	169	274	358	209	168	145	119	265	87	306	*Type I*	Howe and Sibley, 1995
ENT[a] (TgH 00004)	-	-	-	-	-	-	-	-	209	166	145	119	267	87	308	*Type I*	Dardé et al., 1992
B1[a] (TgA 103001)	-	-	-	-	-	-	-	-	209	166	147	119	273	87	306	*Type I*	Canada et al., 2001
JONES[a]	289	242	207	158	336	169	274	356	235	174	142	111	265	97	310	*Type II*	Mercier et al., 2010
Me49 (TgA 00001)	-	-	-	-	-	-	-	-	215	174	142	111	265	91	310	*Type II*	Lund and Jacobs, 1983
PRU[a] (TgH 00001)	-	-	-	-	-	-	-	-	209	176	142	117	265	121	310	*Type II*	Ajzenberg et al., 2002
CTG	289	242	205	160	336	165	278	356	215	190	147	111	269	89	312	*Type III*	Pfefferkorn and Kasper, 1983
NED[a] (TgH 00003)	-	-	-	-	-	-	-	-	209	190	147	111	267	91	312	*Type III*	Dardé et al., 1992
VEG (TgH 00005)	-	-	-	-	-	-	-	-	213	188	153	111	267	89	312	*Type III*	Howe and Sibley, 1995

Reference strains: Type I (GT1, ENT, and B1), Type II (JONES, Me49 and PRU), and classical Type III (CTG, VEG and NED)

- : identical to the previous value (located above); NA: not amplified.

[a]: these reference strains were obtained from Biological Resource Centre for *Toxoplasma* (http://www.toxocrb.com).

[b]: allelic polymorphism of MS markers is expressed as sizes of PCR products (bp).

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