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(Article begins on next page)
Wild ungulates as sentinel of BTV-8 infection in piedmont areas

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Abstract:

Bluetongue caused by the serotype 8 virus (BTV-8) appeared for the first time in BTV free areas in northern Italy in 2008. The presence of domestic animals outbreaks, abundant wild ungulates populations, and ongoing regional BTV control plans, made this area interesting to evaluate the role of wild ruminants in BTV-8 epidemiology. We analyzed spleen samples from hunted red deer (Cervus elaphus), roe deer (Capreolus capreolus) and Alpine chamois (Rupicapra rupicapra) by quantitative RT-PCR. Samples were collected from 2008 to 2011 in two provinces of Piedmont region. BTV-8 was detected in all ungulate species, confirming their receptivity to the infection. However, the viral load in the positive specimens was low, and decreased from 2008 to 2011. These results, together with the extinction of the epidemic following a regional livestock vaccination campaign, lead to hypothesize that wild ungulates were an epiphenomenon and that they had not an important role in the domestic transmission cycle of BTV-8 in the area. In spite of this, wild ruminants appear to be good sentinels of BTV circulation and their monitoring could be useful for surveillance in piedmont areas.
Bluetongue (BT) is an infectious disease of ruminants caused by a RNA virus (BTV) belonging to the Reoviridae family, genus Orbivirus. The virus may infect various ruminant species, although clinical disease and mortality are generally associated with sheep (Erasmus, 1990). It is characterized by a high genetic variability, and 26 serotypes (including the new Toggenburg and Kuwait serotypes; Chaignat et al., 2009; Maan et al., 2011) have been recognized so far. Midges of the genus Culicoides (Diptera: Ceratopogonidae) are the vectors of BTV, and their distribution determines the geographic diffusion of the disease (Maclachlan et al., 2011).

BT is a reportable disease with high socioeconomic impact, affecting the international trade of animals and animal products (Saegerman et al., 2008). It was considered an exotic disease with probable African origin until 1998, when the virus began its spread in Europe (Eschbaumer et al., 2009). After 2006, some BTV serotypes expanded across the 45° parallel (Saegerman et al., 2008). The most important outbreaks in central and northern Europe were caused by BTV-8. This serotype showed peculiar characteristics, including an unexpected virulence in cattle (Thiry et al., 2006).

In Italy serotypes 2, 4, 9, and 16 have been reported since 2000 in central and southern regions. In 2008 BTV-8 appeared in the north, in Veneto and Piedmont regions, and in Sardinia, while BTV-25 was present in Lombardia region, close to the Swiss borders (Sozzi et al., 2009).

Piedmont region was a previously BTV free area in north-western Italy (Fig. 1). BTV-8 infection was firstly reported in a cow during October 2008 in Cuneo province (Fig. 2A), from a sentinel farm included in the Italian BTV control program (Caporale and Giovannini, 2010). The farm had a mixed breeding of cows, sheep and goats, and two bulls had been introduced in 2004 and 2007 from France. Seven days after the removal of the infected cow, a 6 months-calf born in the same farm was found positive. In the following months of 2008, other infections caused by BTV-8 were
recorded in neighboring areas, for a total of 16 positive farms. In 2009 and at the beginning of 2010, ten new positive farms were discovered in Turin and Asti provinces\(^1\) (Fig. 2A). Most BTV-8 outbreaks occurred at the foothills of the Alps, in areas characterized by woodland and low zootecchnical density, where domestic animals are sometimes free-ranging. In Piedmont, culicoides of the *Obsoletus* and *Pulicaris* complexes are present (Goffredo et al., 2004).

Various measures to limit the spread of the disease were adopted by the Piedmont veterinary services, in relation to a local risk analysis, including restrictions to the movement of susceptible animals and the implementation of two vaccination campaigns in domestic ruminants. The performed activities prevented the damages of the disease, maintained the trade flows and reduced the spread of the infection, with the last goal of disease eradication\(^2\).

Different hypothesis were proposed to explain the introduction of BTV in Piedmont. First, the importation of infected animals from France; indeed, around 180000 heads of cattle are imported every year in Piedmont, mainly to fattening farms\(^3\). However, also the free movements of infected wild animals and vectors from neighboring countries (France and Switzerland) could be implicated. BTV-8 was indeed widespread in France during the late summer of 2008, and cases had been reported in areas close to the Italian borders. The existence of interconnected domestic and wild cycles that could account for the maintenance of BTV has been hypothesized (Ruiz-Fons et al., 2014).

In this study, we employed RT-qPCR to analyze tissue samples from red deer, roe deer and chamois captured during the 2008-2011 hunting seasons in Piedmont, to evaluate the role of wildlife during the BTV-8 epidemics in the region. Determining such role could be very important for the implementation of surveillance plans and to face future BTV epidemics.

\(^1\) [http://www.regione.piemonte.it/sanita/cms/documentazione/category/96-2011.html](http://www.regione.piemonte.it/sanita/cms/documentazione/category/96-2011.html)


\(^3\) National husbandry register. Statistics. [http://statistiche.izs.it/portal/page?_pageid=73,12918&_dad=portal&_schema=PORTAL](http://statistiche.izs.it/portal/page?_pageid=73,12918&_dad=portal&_schema=PORTAL)
Materials and methods.

Study area.

Piedmont is located in north-western Italy (44°23’0’’ N, 7°33’0’’ E; Fig.1). It is an Alpine region characterized by an important livestock farming system. According to 2013 statistics, cattle heads are estimated at around 778000 (13% of Italy) and small ruminants at 188000 (2% of Italy\(^3\)). Our study focused in Cuneo and Torino provinces (Fig. 1), in which the main domestic BTV-8 outbreaks occurred in 2008-2009 (Fig. 2a). Sampling was performed in hunting districts in the alpine valleys. In these areas, livestock is usually brought to pasture during summer months. Animals were captured at an altitudinal range comprised between 300 and 1600 m a.s.l. (above sea level). Moreover, hunted red deer from an enclosed regional natural park in the periurban area of Torino (La Mandria, 265 m above sea level; Fig.1) were sampled. Livestock breedings are located in close proximity of the park walls.

The regional authorities coordinate wildlife censuses and control hunting in the region. The hunting quotas, based on ungulates numbers which are compatible with the agro-sylvatic and pastoral activities, were set at 0.5-1 red deer, 5-10 roe deer, and 6 chamois per 100 ha\(^3\). A different management is adopted in 'La Mandria' park, in which the red deer density is at present estimated at 14 red deer per 100 ha.

Animal tissues collection.

Samples were collected during the hunting seasons in the period March 2008 - June 2011 from red deer (Cervus elaphus), Alpine chamois (Rupicapra rupicapra) and roe deer (Capreolus capreolus).

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\(^3\)www.regionepiemonte.it
Ungulates were brought from hunters to hunting lodges and spleen were collected and stored at -20°C until analysis. Spleen was the target organ of choice for maximizing the likelihood of detecting BTV RNA by molecular analysis (López-Olvera et al., 2010; Worna et al., 2010; Rossi et al., 2014), since sample collection was performed in the field and it was occasionally carried out by hunter. Species, sex, age, location and hunting date of animals were recorded. The animals were classified as juveniles (less than 1 year old) or adults (equal or more than 1 year old). Aging was based on tooth replacement and corn.

**Molecular analyses.**

*RNA extraction.* RNA was extracted by using Trizol reagent (Invitrogen) from 100 µg tissue, in accordance with the manufacturer’s protocol, and quantified with NanoDrop 2000 instrument (Thermo Scientific).

*Real time RT-PCR (RT-qPCR).* The primers targeting the NS3 gene of BTV were designed based on ClustalW multiple alignment of GeneBank reference sequences of BTV genotypes circulating in Italy or at risk of introduction (1, 2, 4, 8, 9 and 16). The RT-qPCR assay was performed using 100ng of RNA extracted from the samples. The 25µl reaction mix contained 5µl of RNA extract in RNase and DNase-free water, 2XQuantiFast RT-MIX SYBR Green (Qiagen), 0.03 mM concentrations of both primers (forward primer: 5'-TAT CCG GGC TGA TYC RAA GG -3'; and reverse primer: 5'-GCG TAC GAT GCG AAT GCA GC-3'). The mix of primers and template was denatured at 95°C for 5min and subsequently added at the reaction mix. Cycling condition included: retrotranscription at 50°C for 30min, denaturation at 95°C for 5 min; 50 cycles of 95°C for 10s, 60°C for 30s. Post-amplification melting temperature (Tm) analysis from 50 to 95°C at 0.5°C increments was conducted to determine specific NS3 product (Tm ~ 83.6 °C) (Applied Biosystems 7300 Real Time PCR System). The Applied Biosystems detection software was used to determine threshold cycle.
(Ct) values, Tm, and the standard curve. Negative controls, including RNase and DNase-free water and nucleic acid extracts from sure negative samples, were used to determine any possible cross-reactivity or contamination (false-positive results). RNA from a BTV-2 positive sample was used as positive control. All experiments were run in duplicate and were replicated twice. ATPase gene amplification was included as a control, in order to detect any inhibition in the BTV detection.

Some positive samples were sequenced to confirm the results (BMR genomics, Padova, Italy). The chromatograms of the nucleotide sequences were submitted to BLAST analysis.

**Standard curve.** For calibration of the RT-qPCR assay, we cloned the BTV-8 amplicons into pCR-XL-TOPO cloning vector (Life Technologies). The identity of the insert was confirmed by sequencing. After plasmid preparation, we generated a standard curve with known copy numbers of NS3-RNA transcripts from the plasmid, containing the NS3 fragment obtained by PCR. The Riboprobe kit (Promega) was used to produce the standard transcripts, following the ‘Large Amount RNA Synthesis protocol’ provided by the manufacturer. Transcribed RNA was DNase treated, purified using an RNA clean-up protocol (RNeasy MinElute Cleanup Kit, Qiagen) and extracted using Trizol (Life Technologies). The purified RNA was quantified by use of Nanodrop and the corresponding copy number was calculated. Calibration curves were generated by amplification of 100 fold serial dilutions of $10^7$ to $10^1$ RNA copies per reaction. Dilutions were also used to test the sensitivity limits of the assay: a log-10 titration series of in vitro transcribed RNA indicated that less than 10 copies of BTV NS3 gene segment ssRNA could be detected.

**Statistical analysis:**

Prevalence of PCR-positive results were calculated, with 95% exact binomial confidence intervals (95%CI), per hunting season, animal species, sex and study area. The hunting season was considered as starting from September each year. The Fisher’s Exact test was used to study the
association among categorical variables. Mean and standard deviation of Ct levels of positive
samples were calculated by hunting season year. Analyses were performed by R software (R
Development Core Team 2008).

Results:
A total of 235 animals were sampled: 102 red deer, 78 roe deer, and 55 chamois. Most of the
samples were collected during the hunting seasons 2009-2010 (n=135; from 48 red deer, 43 roe
deer, 44 chamois) and 2010-2011 (n=84; from 51 red deer, 24 roe deer, 9 chamois). Other 16
samples were collected during the 2008-2009 hunting season (from 3 red deer, 11 roe deer, 2
chamois).

Due to the convenience sampling, samples distribution by species was not homogeneous in the
study areas: most of red deer samples (92.2%) were collected in Torino province, while the
majority of roe deer (91.0%) and chamois (89.1%) were sampled in Cuneo.

Spleen samples were submitted to RT-qPCR analysis. BTV NS3 gene was detected in 15.7% of the
animals (n=37, 95% CI 11.3-21.0): 21 red deer, 12 roe deer and 4 chamois. The threshold cycle (Ct)
RT-PCR values ranged between 23.15 and 35.2, with a mean Ct value of 30.4±2.6. The melting
curve analysis revealed that positive samples belonged to BTV serotype 8. To confirm the results,
three samples from red deer were sequenced: one from Torino valley (hunting season 2008-2009),
one from Cuneo valley and one from 'La Mandria' park (hunting season 2009-2010). The
sequences showed a similarity of 99.6%, 98.9% and 99.5% to BTV-8 respectively.

Infection prevalence by animal species, sex, age, hunting season, and province of collection, is
shown in Table 1. No difference in the BTV prevalence was detected among ungulate species
(Fisher Exact test; p=0.09). However, red deer were the most infected species in all sampled areas.
Indeed, in Cuneo province, three out of eight examined red deer were infected (37.5%; 95%CI: 8.5-
whereas only 8.2% (95%CI: 2.3-19.6) of chamois and 16.9% (95%CI: 9.0-27.7) of roe deer resulted positive. In Torino province, only red deer were found infected; however, only a small number of chamois (n=7) and roe deer (n=6) was examined.

The age class and sex of the animals were not significantly associated with PCR positivity (p=0.15 and p=0.8 respectively). The infection prevalence was significantly different among hunting years (p=0.034), with BTV prevalence decreasing from 2008 to 2011 (Table 1). Moreover, positive samples from 2008-2009 showed lower mean Ct values (28.5±2.7) compared to the other hunting seasons (30.6±2.7 in 2009-2010, and 30.6±1.8 in 2010-2011). Most of the infected animals (n=26) were hunted during late summer-autumn, but some positivities were also registered in wintertime and early spring (December to June).

BTV prevalence was similar in the two Piedmont provinces (Table 1). When considering red deer only, no difference in the infection prevalence between La Mandria (25%; 95%CI: 14.3-38.4) and the Alpine valleys (15.2%; 95%CI: 6.3-28.9) was recorded (p=0.3), while a significant difference (p=0.036) was found when considering all animal species (prevalence in the Alpine valleys: 12.8%; 95%CI: 8.3-18.6). Positive animals were recorded in Cuneo from April 2008 to October 2010, and were captured in municipalities located from 480 m to 1650 m a.s.l. In Torino province, the positive animals were captured from October 2009 to October 2010 in municipalities located between 260 and 1600 m a.s.l. Fig. 2 shows BTV-8 domestic outbreaks (A), and municipalities in which wild ungulates were tested (B) in 2008-2010 in Piedmont. The geographic pattern suggests a temporal association in the same areas between domestic and wildlife positivities, with positivity in wildlife following with one-year delay the domestic outbreaks. No animals captured between October 2010 and July 2011 (n=26) resulted infected.

**Discussion:**
Our study confirms the wild ungulates receptivity to BTV-8 infection (García-Bocanegra et al., 2011; Linden et al., 2010; Rossi et al., 2010, 2013).

As reported in other studies, we detected a low viral load in the positive spleens, which could indicate a very low or absent viral load in blood and skin (Worna et al., 2010). Our positive ungulates were probably not able to infect biting midges, at least in the moment they were hunted. Indeed, experimental infections in red deer indicated that, although the BTV RNA is detectable up to 112 days post infection (dpi), the isolation from skin samples was only successful during the viraemic peak at 14 dpi, which corresponds to the peak of BTV RNA detection by RT-qPCR in blood. This demonstrated that the infectious agents can be transmitted to the midges only during the viraemic peak of infection (López-Olvera et al., 2010). BTV-8 experimental infection in calves, where the virus could be detected up to 157 days, showed that real time RT-PCR remains positive although no circulating virus is detectable in the peripheral circulation (Di Gialleonardo et al., 2011). RT-PCR is thus a valuable method to estimate recent BTV infections in wildlife (Rossi et al., 2013), but it is not suitable to assess the time at risk in which culicoides can be infected, due to the discrepancy between the time in which virus isolation is possible and RNA is amplified by RT-qPCR (Worwa et al., 2010). Moreover, the minimal level of viraemia in a host, necessary to infect the culicoides, is not known. Although virus titers in blood lower than $10^3$ TCID$_{50}$ (median tissue culture infective dose)/ml have traditionally been considered a safe threshold, insects acquiring BTV from animals with lower viraemic titers have been reported (Savini et al., 2008). However, a high BTV-8 viral concentration in the bloodmeal of artificially fed midges resulted in absence of infection after the insect extrinsic incubation period (Del Rio López et al., 2012).

We observed a slightly higher BTV prevalence in red deer compared to the other species, although this difference was not significant. In France, high RT-PCR prevalence for BTV1 and BTV-8 were reported in red deer (77.8% and 37.8% in 2008 and 2009, respectively), while BTV-8 was not
detected in three roe deer and one Alpine chamois (Rossi et al., 2013). Also serological studies report the highest seroprevalences in red deer (up to 66.3%) and fallow deer (*Dama dama*; up to 50%), both belonging to the *Cervinae* subfamily (Corbière et al., 2012; García-Bocanegra et al., 2011; Linden et al., 2008; Linden et al., 2010; Rossi et al., 2010 and 2013; Ruiz-Fons et al., 2008). Roe deer were generally found sero-negative (Boadella et al., 2010; Corbière et al., 2012) or with sero-prevalences up to 5.1% (Linden et al., 2010; Rossi et al., 2010; Ruiz-Fons et al., 2008). Scarce information can be found for the Alpine chamois, which were found sero-negative by Corbière et al. (2012) and Rossi et al. (2010). Antibodies to BTV were also detected in Europe in mouflons (*Ovis aries musimon*), Spanish ibex (*Capra pyrenaica*) and ibex (*Capra ibex*) (Falconi et al., 2011).

In Europe, red deer is considered the most relevant wild ungulate host for BTV (Ruiz-Fons et al., 2014). Indeed, red deer can maintain the viral RNA for long periods, remaining essentially asymptomatic (Falconi et al., 2011), and populations are abundant. Areas with higher red deer densities in Spain have been shown to display higher BTV prevalence (Garcia et al. 2009); high animal densities can indeed favor the effective contact rate for the infected culicoides. In our study areas, red deer populations have increased their abundance in the past decades. Although a higher infection prevalence was detected in red deer living in a high density area (La Mandria) compared to the Alpine valley populations, the difference in infection prevalence between the different areas was not significant. The high infection prevalence in La Mandria could be due to the close proximity to the park walls of some livestock breedings which were found positive to BTV-8 in 2009, since infected insects can easily fly between the external area and the park. A significant difference in infection prevalence was instead recorded when considering all animal species, probably because of the lower prevalence in mountain ungulates such as the chamois, as observed in France (Rossi et al., 2013).
Sex and age did not appear to be risk factors for BTV infection in our study. BTV association with these factors are not consistent throughout literature (Ruiz-Fons et al., 2014).

We observed a progressive viral load reduction in the positive samples collected from 2008 to 2011. This could be associated to a lower viral circulation in Piedmont region, which occurred after the 2009 and 2010 vaccination campaigns: the reduced infection burden in domestic animals could have determined a consequent lower prevalence in biting midges and wildlife. Indeed, we did not detect infection in ungulates after October 2010. The finding of positivities in early winter and spring could be a result of persistent infections.

The temporal association between the positivity in domestic and wild animals is noteworthy. Indeed, the first domestic outbreaks occurred in Cuneo in 2008 and in Torino in 2009, and we found positive wild ungulates in Cuneo from 2008 onwards, and in Torino from 2009 onwards (Fig. 2). Wildlife infection thus followed the domestic animal infection trend. As observed in France (Rossi et al., 2013), BTV was maintained in wild ungulates in periods characterized by negligible livestock infections.

All these data may suggest that wild ungulates represented an epiphenomenon, and did not have an important role in the domestic transmission cycle of BTV-8 in Piedmont. This thesis is confirmed by the ‘natural experiment’ which occurred in the region one year after the end of the compulsory vaccination campaign: no BT cases or laboratory positivity were registered from 2011 to date in domestic animals, indicating that the virus was not re-introduced nor it overwintered in the region. Also in seroprevalence studies, a decline in BTV seroprevalence in red deer was shown after an epidemic event, or domestic livestock vaccination (Corbière et al., 2012; A. Linden et al., 2010; Stallknecht et al., 1991), suggesting that red deer are not involved in the maintenance of BTV for long periods (Falconi et al., 2011).
Our results confirm the serological positivities (3 out of 49 animals) observed in hunted red deer from La Mandria in 2009-2010 (Radaelli et al., 2011); in this study low antibody titres were recorded, and the seroneutralization test confirmed that the involved serotype was BTV-8.

Our work contributes to the knowledge on the role of wild ungulates in BTV-8 epidemiology in north-western Italy, although further studies are needed to identify the factors that influenced the virus dynamics in wildlife. Even though the role of wildlife as BTV reservoir in Piedmont is unlikely, these animals serve as feeding source for culicoides and can be considered good sentinels of infection (Falconi et al., 2011; Stallknecht and Howerth, 2004). Samples collection and testing, indeed, is particularly easy in areas where wild ungulates are routinely subjected to population control plans and hunted. Wild ungulates could thus be very useful for BTV surveillance in areas with promiscuous presence of wild and domestic animals.

Conflict of interest
No competing financial interests exist.

Acknowledgements
We thank Prof. Ezio Ferroglio, Università di Torino, for kindly providing some precious samples. We thank the personnel of the Centro Multizonale in Borgo San Dalmazzo for helping in animal samples collection. Thanks to Eleonora Facchinelli for providing regional data for the preparation of maps. This study was funded by the Regione Piemonte, Progetti di Ricerca Sanitaria Finalizzata.

Bibliographic references


Table 1. Prevalence of BTV infection in ungulates hunted in Piedmont in 2008-2011

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<td>(n, 95% CI)</td>
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<td><strong>Species</strong></td>
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<tr>
<td>Red deer</td>
<td>102</td>
<td>20.6% (21, 13.2-29.7)</td>
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<tr>
<td>Roe deer</td>
<td>78</td>
<td>15.4% (12, 8.2-25.3)</td>
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<tr>
<td>Alpine chamois</td>
<td>55</td>
<td>7.3% (4, 2.0-17.6)</td>
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<td><strong>Hunting season</strong></td>
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<tr>
<td>2008-2009</td>
<td>16</td>
<td>25% (4, 7.3-52.4)</td>
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<tr>
<td>2009-2010</td>
<td>135</td>
<td>19.3% (26, 13-26.9)</td>
</tr>
<tr>
<td>2010-2011</td>
<td>84</td>
<td>8.3% (7, 3.4-16.4)</td>
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<td><strong>Age</strong></td>
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<tr>
<td>&lt;1 year</td>
<td>59</td>
<td>16.9% (10, 8.4-29.0)</td>
</tr>
<tr>
<td>&gt;1 year</td>
<td>176</td>
<td>15.3% (27, 10.4-21.5)</td>
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<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>119</td>
<td>19.3% (23, 12.7-27.6)</td>
</tr>
<tr>
<td>Male</td>
<td>116</td>
<td>12.1% (14, 6.8-19.4)</td>
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<td><strong>Province</strong></td>
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<tr>
<td>Cuneo</td>
<td>128</td>
<td>14.8% (19, 9.2-22.2)</td>
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<tr>
<td>Torino</td>
<td>107</td>
<td>16.8% (18, 10.2-25.3)</td>
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**Fig. 1.** Map of Piedmont region with administrative provinces with BTV-8 outbreaks in 2008-2010 (A= Torino, B= Cuneo, C= Asti; circle= La Mandria park) and elevation (meters above sea level, a.s.l.).
Fig. 2. BTV-8 domestic outbreaks (A) and tested municipalities for BTV presence in wildlife (B; gray = negative, black = positive for at least one tested animal) in Piedmont region, north-western Italy, in 2008-2010.