Title Page

(i.) Full title

Detection of the European epidemic strain of *Trichomonas gallinae* in finches, but not other non-columbiformes, in the absence of macroscopic disease

(ii.) Author names

Erasmus zu Ermgassen[a1], Chris Durrant[a2], Shinto John[a], Roxanne Gardiner[a], Abdulwahed F. Alrefaei[b], Andrew A. Cunningham[a], Becki Lawson[a*]

(iii.) Affiliations

[a] Institute of Zoology, Zoological Society of London, Regent's Park, London NW1 4RY, U.K.

[b] University of East Anglia, School of Biological Sciences, Norwich NR4 7TJ, U.K.

[1] Department of Zoology, University of Cambridge, Downing Street, Cambridge CB2 3EJ, U.K.

[2] Queen Mary University of London, Fogg Building, Mile End Road, London, E1 4NS, U.K.

(iv.) Running title

Trichomonas gallinae in finches without necrotic ingluvitis

(iv.) *Corresponding author:

Becki Lawson, Institute of Zoology, Zoological Society of London, Regents Park, London, NW1 4RY, UK

Email: becki.lawson@ioz.ac.uk

Tel: +44 207 449 6677

Summary

Finch trichomonosis is an emerging infectious disease affecting European passerines caused by a clonal strain of *Trichomonas gallinae*. Migrating chaffinches (*Fringilla coelebs*) were proposed as the likely vector of parasite spread from Great Britain to Fennoscandia. To test for such parasite carriage, we screened samples of oesophagus/crop from 275 Apodiform, Passeriform and Piciform birds (40 species) which had no macroscopic evidence of trichomonosis (i.e. necrotic ingluvitis). These birds were found dead following the emergence of trichomonosis in Great Britain, 2009-2012, and were examined post-mortem. Polymerase chain reactions were used to detect (ITS1/5.8S rRNA/ITS2 region and single subunit rRNA gene) and to subtype (Fe-hydrogenase gene) *T. gallinae*. *Trichomonas gallinae* was detected in six finches (three chaffinches, two greenfinches (*Chloris chloris*) and a bullfinch (*Pyrrhula pyrrhula*)). Sequence data had 100% identity to the European finch epidemic A1 strain for each species. While these results are consistent with finches being vectors of *T. gallinae*, alternative explanations include the presence of incubating or resolved *T. gallinae* infections. The inclusion of histopathological examination would help elucidate the significance of *T. gallinae* infection in the absence of macroscopic lesions.

Key words: trichomonosis, passerine, epidemiology, wild bird, emerging infectious disease

Key findings:

- PCR detected *T. gallinae* in six finches with no gross evidence of necrotic ingluvitis.
- No *T. gallinae* infection detected in non-Fringillid passerine species tested.
- These findings are consistent with the hypothesis that the chaffinch acts as a parasite vector.

Introduction

Trichomonas gallinae is a protozoan parasite known to cause morbidity and mortality in columbiforms, birds of prey, and less frequently in passeriform and psittaciform species (Amin *et al.*, 2014). Birds with trichomonosis show non-specific clinical signs of malaise (e.g. lethargy and fluffed-up plumage), sometimes in combination with dysphagia, which occurs as a result of necrotic pharyngitis and/or ingluvitis. Parasite transmission occurs through contact with fresh saliva, either directly through conspecific feeding (e.g. during courtship or when feeding young), or indirectly at contaminated water and food sources (Forrester and Foster, 2009).

Whilst isolated cases of trichomonosis in finches have been diagnosed in Great Britain (GB) since the early 1990s (Lawson *et al.*, 2012), finch trichomonosis was identified as a significant emerging infectious disease (EID) in 2005 (Robinson *et al.*, 2010). A single clonal strain of *T. gallinae* (Lawson *et al.*, 2011a) caused epidemic mortality of both greenfinches (*Chloris chloris*) and chaffinches (*Fringilla coelebs*) in subsequent years (Lawson *et al.*, 2012). This EID caused a 35% population decline of breeding greenfinches across GB from 2006-09 (from ca 4.3 million to ca 2.8 million birds), with a concomitant 50% reduction of the maximum mean number of greenfinches (a proxy for flock size) visiting gardens (Lawson *et al.*, 2012). Greenfinch and chaffinch represent the species most frequently diagnosed with finch trichomonosis. Since its epidemic emergence, the disease has also been confirmed in a range of other passerines, comprising Emberizidae (yellowhammer *Emberiza citrinella*), Fringillidae (Brambling *Fringilla montifringilla*, bullfinch *Pyrrhula pyrrhula*, goldfinch *Carduelis carduelis*, siskin *Carduelis spinus*), Paridae (great tit *Parus major*), Passeridae (house sparrow *Passer domesticus,* tree sparrow *Passer montanus*), Prunellidae (dunnock *Prunella modularis*) and Turdidae (blackbird *Turdus merula*) (Robinson et al., 2010; authors' *unpublished data*).

Molecular investigation has found evidence of *T. gallinae* strain diversity in columbiform hosts in GB; however, infection with the same clonal strain of the parasite affecting finches is predominant in

British non-passerine species comprising pigeons, doves and birds of prey (Chi *et al.*, 2013). Finch trichomonosis is hypothesised to have emerged as a result of *T. gallinae* spill-over from columbiform to passeriform hosts in GB. Whilst it remains speculative, this spill-over may have occurred at a shared feeding site(s), such as domestic gardens with bird feeders (Lawson *et al*. 2012). After emergence in GB, finch trichomonosis spread to continental Europe, with incidents first confirmed in Fennoscandia in 2008 (Neimanis *et al.*, 2010) before its spread to central Europe (Peters *et al.*, 2009; Ganas *et al.*, 2014). Examination of epidemiological and ring recovery data indicated migrating chaffinches as the most likely vector, since they overwinter in GB before moving in large numbers to their summer breeding grounds in Fennoscandia with autumn passage through the northern coastline of western Europe on their return journey (Lawson *et al.*, 2011b).

While *T. gallinae* can cause morbidity and mortality in wild columbiforms (Forrester and Foster, 2009), the majority of columbiform infections are aclinical (i.e. without disease) or subclinical (i.e. without observed clinical signs) with the outcome of infection influenced by factors such as parasite strain virulence and host immunity (Stabler 1961; Kocan & Knisley 1970; Forrester & Foster 2009). Given the sometimes high rates of *T. gallinae* infection without apparent disease (i.e. trichomoniasis) detected in wild columbiforms and the suspected spread of finch trichomonosis by chaffinch migration from GB to Fennoscandia and central Europe, we hypothesised that chaffinches may also carry *T. gallinae* without showing clinical signs thus enabling parasite spread. Since *T. gallinae* is a labile parasite killed by desiccation and is incapable of long term environmental persistence (surviving only short periods in water and up to five days in moist grain (Forrester and Foster, 2009; Gerhold *et al.*, 2013; Purple *et al.*, 2015)), its movement by wild birds is likely to play an important role in the epidemiology of this parasitic infection.

Here we present findings from a polymerase chain reaction (PCR)-based survey of upper alimentary tract (crop and/or oesophagus) samples from 275 garden birds (Apodiformes, Passeriformes and

Piciformes) found dead in the UK over a 3-year period (May 2009-July 2012) following the epidemic emergence of finch trichomonosis in GB. Birds were examined post mortem and samples were selected from cases with no evidence of necrotic ingluvitis in order to investigate whether the parasite was present in passerines with no macroscopic disease.

Materials and methods

Sample selection:

Wild bird carcasses found by members of the public within the United Kingdom were submitted to a national scheme for infectious and non-infectious disease surveillance of wild birds (Robinson *et al.*, 2010). Post-mortem examinations (PMEs) were conducted following a standardised protocol comprising systematic external and internal inspection of organ systems, with microbiology, parasitology and histology performed as indicated by the presence of macroscopic lesions (Robinson *et al.*, 2010). Culture of *T. gallinae* was attempted from the majority of Passeriform submissions when the carcass had not been frozen: oesophagus/crop tissue samples and/or swabs were inoculated into Oxoid Trichomonas Medium No. 2, incubated at 30^{o}C and checked at 1,2 and 5 days for evidence of motile parasites (Robinson *et al.*, 2010). Tissue samples from a range of organs, including crop and/or oesophageal tissue, were routinely collected and stored at - 80°C. A case definition was utilised for finch trichomonosis, based on detection of macroscopic necrotic ingluvitis lesions with diagnosis confirmation using parasite culture and/or PCR (Robinson *et al.*, 2010) .

We selected available frozen crop/oesophagus samples from Apodiform, Passeriform and Piciform species that were examined post mortem between May 2009 and July 2012, during the finch trichomonosis epidemic in GB, and which had no observed macroscopic lesions characteristic of finch trichomonosis.

DNA extraction:

DNA was extracted from crop/oesophageal tissue using either the DNeasy Blood and Tissue Kit (Qiagen, UK) or Isolate DNA Kit (Bioline, UK) according to the manufacturers' instructions. To test for possible DNA cross-contamination, a DNA extraction negative control was included every 24 samples. All DNA extracts were screened using PCR regardless of DNA concentration. It was possible to obtain PCR positives that generated high quality sequence data even for samples with a low DNA concentration.

ITS1/5.8S rRNA/ITS2 region PCR:

A PCR was used to amplify the ITS1/5.8S rRNA/ITS2 region (henceforth ITS region) using the published primers (TFR1 forward - TGCTTCAGTTCAGCGGGTCTTCC and TFR2 reverse - CGGTAGGTGAACCTGCCGTTGG) (Gaspar da Silva *et al.*, 2007). Modifications were made to published PCR protocols (Robinson et al., 2010; Chi et al., 2013): reactions were run with 7.5 µl HotStarTaq plus Master Mix (Qiagen, UK), 2 µl of 10 pg/µl forward and reverse primer, 3.5 µl of molecular grade water, and 1 µl of extracted DNA to complete a 16 µl reaction mix. Samples were run on a GeneAmp PCR System 2700 (Applied Biosystems, UK) using the following temperature regime: 94°C for 10 minutes initial denaturation, followed by 45 cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute; final elongation took place at 72°C for 10 minutes. Each PCR run included a PCR positive control (DNA extracted from a *T. gallinae* culture), a DNA extraction negative control (to confirm reagent negativity and the absence of cross-contamination during DNA extraction) and a PCR negative control consisting of molecular grade water.

Single subunit (SSU) rRNA gene PCR:

In order to increase the sensitivity of detection, a nested PCR for the trichomonad small subunit (SSU) rRNA (or 18S rRNA) gene was also performed. The initial PCR was carried out using primers SSU-Fwd (TACTTGGTTGATCCTGCC) and SSU-Rev (TCACCTACCGTTACCTTG), as per Robinson et al., (2010). PCR reactions were run with 5 µl HotStarTaq plus Master Mix; 1.5 µl of 10 pg/µl forward and

reverse primer, 2µl of molecular grade water, and 2 µl of 1:10 diluted DNA. The temperature regime used an initial denaturation temperature of 95°C for 5 minutes, followed by 35 cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute; final elongation took place at 72°C for 10 minutes. Four µl from this initial PCR were then added to 3µl of forward and reverse primers (TN3 forward – ATAGGACTGCAAAGCCGAGA and TN4 reverse-TGATTTCACCGAGTCATCCA); 10µl HotStarTaq plus Master Mix and 4µl of molecular grade water. The temperature regime used was 95°C for 5 minutes initial denaturation, followed by 40 cycles of 94°C for 1 minute, 57°C for 1 minute, and 72°C for 1 minute; final elongation took place at 72°C for 10 minutes.

PCR product visualisation and sequencing:

Amplified PCR products were run on an ethidium bromide or GelRed™ (Biotium) stained 2% agarose gel alongside a GelPilot 1000bp Ladder (Qiagen, UK). Bands were visualised using a transilluminator. PCR products of the expected size (ca. 335 bp for ITS region PCR and ca 149 for SSU rRNA gene nested PCR) were sequenced for confirmation using a 3130XL ABI sequencer using BigDye® Terminator v3.1 sequencing kit (ABI). Sequence data were aligned in both directions for each sample using MEGA 5.0 software (Tamura *et al.*, 2013) and were compared with available gene sequences within NCBI Genbank using the BLAST search function to determine species identification within the Trichomonadidae.

Samples were considered positive for *T. gallinae* on the basis of sequence data from amplification of either the ITS region and/or SSU rRNA gene. ITS region PCR was repeated for any samples negative on the first attempt if an SSU rRNA gene PCR product was obtained and confirmed as *T. gallinae* on sequencing.

Fe-hydrogenase gene PCR:

In order to subtype *T. gallinae*, we conducted PCR for the Fe-hydrogenase gene on samples positive

for the parasite on ITS region and/or SSU rRNA gene PCR and sequencing. This was limited to positive samples since amplification of the single-copy Fe-hydrogenase gene can be challenging in DNA extracted from tissue rather than parasite culture (Chi *et al.*, 2013). The primers ((TrichhydFOR (GTTTGGGATGGCCTCAGAAT) and TrichhydREV (AGCCGAAGATGTTGTCGAAT)) and protocol from Lawson *et al.* (2011a) were used with the following modifications: reactions were run with 10µl BioMix™ (Bioline, UK), 3 µl of 10 pg/µl forward and reverse primer, 2 µl of molecular grade water, and 1 µl of extracted DNA to complete a 19µl reaction mix. Reactions were run using the following temperature regime: 94°C for 15 minutes initial denaturation, followed by 35 cycles of 94°C for 1 minute, 52°C for 30 second, and 72°C for 2 minute; final elongation took place at 72°C for 5 minutes. Each PCR run included a PCR positive control (DNA extracted from a *T. gallinae* culture obtained from a greenfinch) and molecular grade water as a PCR negative control.

Results

Tissue samples that matched our selection criteria were available from 275 wild birds (1 Apodiform, 269 Passeriformes and 5 Piciformes) from 18 different families submitted from across the UK: these comprised 250 from England, 20 from Wales, 1 from Scotland and 4 from Northern Ireland (Table 1). The causes of death determined at PME were: trauma (35%; 95/275), infectious disease (15%; 42/275), predation (11%; 29/275), a combination of infectious disease and either trauma or predation (17%; 48/275), other (0.01%; 3/275), and undetermined (21%; 58/275).

Nested PCR of the SSU rRNA gene amplified product from six finches (Table 1): three chaffinches, two greenfinches and one bullfinch from separate sites in six counties across England (from three regions comprising South West, North East and West Midlands), Wales and Northern Ireland. In all cases, the sequence obtained was homologous to published data from British finches (Genbank HG008106). ITS region PCR amplified product from four of these same finches; in all cases, the sequence obtained was homologous to published data from British finches (Genbank GQ150752).

206 Fe-hydrogenase gene PCR amplified product from two chaffinches and one greenfinch. The
207 sequence of both of these products was homologous to published data from British finches
208 (Genbank JF681136), confirming that they were the A1 finch epidemic strain of *T. gallinae*.
209
210 *Trichomonas gallinae* parasite DNA was detected in a significantly greater proportion of Fringillidae
211 (3/8) than non-Fringillidae passerine species (0/29) examined (χ^2 =7.3, df=1, P=0.007).
212
213 Details of the incident history and pathological examinations conducted for the six PCR-positive
214 finches are presented in Table 2. These birds were found dead across the study period (2010 n=2,
215 2011 n=1, 2012 n=3) from March-September inclusive. They comprised three females, two males
216 and one finch of undetermined sex. Each age category was represented with three adults, one first
217 year, one juvenile and one first year/ adult identified by plumage inspection. The cause of death
218 category assigned on the basis of available pathological findings was trauma for two finches and
219 undetermined for the remaining four finches. Trichomonosis was confirmed at PME in other finches
220 that were found dead and examined from three of these sites and salmonellosis was confirmed in a
221 finch from a fourth site: no concurrent infectious disease was identified at the remaining two sites.
222
223 Culture of *T. gallinae* was attempted at the time of post mortem examination for five of these six
224 finches (their carcasses were examined fresh and not frozen) and was negative in all cases. No
225 formalin-fixed tissue of the upper alimentary tract was available from the finches for
226 histopathological examination.
227
228 **Discussion**
229
230 In this study of wild birds without macroscopic lesions of necrotic ingluvitis, collected during
231 epidemic finch trichomonosis in GB, we found evidence of *T. gallinae* parasite DNA only in finch

species: greenfinch, chaffinch and bullfinch. Nested PCR of the SSU rRNA gene amplified product with sequence identity to *T. gallinae* from six finches, of which four were positive when tested with the ITS region PCR, and three were positive when tested with the Fe-hydrogenase gene PCR. It is not unexpected that not all three PCR tests were positive in these six finches, because the nested PCR (SSU rRNA gene) has a higher sensitivity than standard PCR (ITS region and Fe-hydrogenase) and the single-copy Fe-hydrogenase gene can be problematic to amplify from infected host tissue. The negative culture results do not preclude the presence of a true *T. gallinae* infection, since isolation relies on the presence of viable parasites and five of the six PCR-positive carcasses were in a moderate or advanced state of decomposition at the time of sample collection.

The presence of *T. gallinae* in a greater proportion of Fringillidae than non-Fringillidae passerine species examined is consistent with the hypothesis that finches are vectors of spread of *T. gallinae* to continental Europe (Lawson et al., 2011b; 2012). In the absence of histopathological examinations confirming the presence of parasites in the absence of lesions, however, these results could have arisen from alternative scenarios. Possible explanations for our results are that PCR positive finches: 1. were in the incubation stage of infection and died from an alternative cause before disease developed; 2. had trichomonosis but with only microscopic lesions of necrotic ingluvitis; 3. had resolved *T. gallinae* infection with no viable parasites present (i.e. no active infection); 4.had recently ingested parasite DNA in the absence of active infection; and 5. had carriage of viable *T. gallinae* parasites without any disease developing. Both 2. and 5. would be consistent with the birds being able to spread the parasite over migratory distances, and 1. could be consistent with this, depending on the length of the incubation period.

The cause of death based on macroscopic, parasitological and microbiological examinations was trauma for one chaffinch and one bullfinch and undetermined for the remaining four finches: consequently we cannot exclude the possibility that one or more of these four finches had

trichomonosis which would have been evident on microscopic examination. The bullfinch that died of trauma was in normal body condition; the other finches were thin, indicating that they might have been suffering from disease prior to death, particularly as one of the greenfinches had equivocal evidence of "oesophageal discolouration" and one of the chaffinches had "marginal oesophageal thickening".

Prolonged carriage of *T. gallinae* has been demonstrated over a 20-month period in the pink pigeon *Columba mayeri* (Bunbury *et al.*, 2008); whether this phenomenon occurs in British finches requires investigation. Our understanding of finch trichomonosis could be advanced through live capture and sampling of large numbers of wild finches for the collection of oropharyngeal swabs to be examined using parasite culture and *T. gallinae*-specific PCR (McBurney *et al.*, 2015). In addition, challenge studies of captive greenfinches and chaffinches with the *T. gallinae* A1 finch epidemic strain would enhance our understanding of the pathogenesis and infection outcomes. Captive studies could include the repeated sampling of live, infected, individuals to determine the proportions of each species that develop overt disease and die or recover, successfully clear the infection and become only transient carriers or become aclinical carriers for a significant period of time. The ability of carriers to transmit infection could be assessed using similar protocols to those of Kietzman (1990), who experimentally demonstrated *T. gallinae* transmission through access to shared drinking water in ringed turtle doves (*Streptopelia risoria*).

We detected *T. gallinae* DNA in three British finch species without macroscopic lesions of necrotic ingluvitis. Further research is required to determine the significance of different finch species in the epidemiology and spread of this parasite.

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366 Table 1 – Number of Apodiform, Passeriform and Piciform birds from which oesophagus/crop was

367 tested by PCR for the presence of *Trichomonas gallinae*.

368

Order	Family	Species	Number of birds (Number of geographic locations)	Number of PCR-positive birds* (Number of geographic locations)
Apodiformes	Apodidae	Swift *Apus apus*	1	
Passeriformes	Aegithalidae	Long-tailed tit *Aegithalos caudatus*	4 (4)	
	Bombycillidae	Waxwing *Bombycilla garrulus*	6 (3)	
	Corvidae	Rook *Corvus frugilegus*	4 (4)	
		Jackdaw *Corvus monedula*	2 (2)	
		Magpie *Pica pica*	2 (2)	
		Carrion crow *Corvus corone*	1	
		Jay *Garrulus glandarius*	1	
	Emberizidae	Yellowhammer *Emberiza citrinella*	3 (1)	
		Reed bunting *Emberiza schoeniclus*	1	
	Fringillidae	Greenfinch *Chloris chloris*	20 (18)	2 (2)
		Chaffinch *Fringilla coelebs*	19 (16)	3 (3)
		Siskin *Carduelis spinus*	10 (5)	
		Bullfinch *Pyrrhula pyrrhula*	6 (6)	1 (1)
		Goldfinch *Carduelis carduelis*	6 (6)	
		Hawfinch *Coccothraustes coccothraustes*	1	
		Linnet *Carduelis cannabina*	1	
		Common Redpoll *Carduelis flammea*	1	
	Hirundinidae	Swallow *Hirundo rustica*	7 (4)	
		House martin *Delichon urbica*	2 (2)	
		Sand Martin *Riparia riparia*	1	
	Motacillidae	Pied wagtail *Motacilla alba*	1	
	Muscicapidae	Spotted Flycatcher *Muscicapa striata*	1	
	Paridae	Great tit *Parus major*	40 (35)	
		Blue tit *Cyanistes caeruleus*	17 (14)	
		Coal tit *Periparus ater*	2 (2)	

	Passeridae	House sparrow *Passer domesticus*	20 (15)
		Tree sparrow *Passer montanus*	2 (2)
	Prunellidae	Dunnock *Prunella modularis*	17 (16)
	Sittidae	Nuthatch *Sitta europaea*	2 (2)
	Sturnidae	Starling *Sturnus vulgaris*	22 (6)
	Sylviidae	Goldcrest *Regulus regulus*	2 (2)
		Chiffchaff *Phylloscopus collybita*	1
	Troglodytidae	Wren *Troglodytes troglodytes*	1
	Turdidae	Blackbird *Turdus merula*	22 (20)
		Robin *Erithacus rubecula*	14 (13)
		Song thrush *Turdus philomelos*	4 (4)
		Mistle thrush *Turdus viscivorus*	1
Piciformes	Picidae	Great spotted woodpecker *Dendrocopos major*	5 (5)
		Green woodpecker *Picus viridis*	2 (2)

*Positive on the basis of PCR targeting the ITS region and/ or the SSU gene and/or Fe-hydrogenase gene.

Table 2 Case details and pathological findings for *T. gallinae* PCR-positive finches

Case number	Species	Month/ Year	Location	Sex	Age	Body condition	Carcass condition	Macroscopic PME findings	*Trichomonas gallinae* culture on Bushby media	ITS region	SSU rRNA gene	Fe-hydrogenase gene	Cause of death category	Infectious disease confirmed at PME in other birds from site?
XT0819-10	Bullfinch	Sep-10	Derbyshire, England	Undetermined	Juvenile	Normal	Mild autolysis	Rib fractures and lung congestion/ haemorhage. No upper GIT lesions.	Neg	Neg	Pos	Neg	Trauma	Yes - greenfinch with trichomonosis Sept-10
XT0065-11	Greenfinch	Sep-10	Devon, England	Female	First year/ Adult	Thin	Advanced decomposition	Oesophagus discoloured and full of seed contents, no thickening described. Skull fracture.	Neg	Neg	Pos	Neg	Undetermined	No
XT0714-11	Chaffinch	Jul-11	Glamorgan, Wales	Female	Adult	Thin	Advanced decomposition	Multiple fractures (coracoid, spine, leg). "Marginally thickened" oesophagus.	Neg	Pos	Pos	Neg	Trauma	No
XT0212-12	Chaffinch	Mar-12	Gwynedd, Wales	Male	Adult	Thin	Moderate decomposition	Black fluid GIT contents; no upper GIT lesions.	ND	Pos	Pos	Pos	Undetermined	Yes – siskin with salmonellosis March-12

XT0232-12	Greenfinch	Mar-12	County Antrim, Northern Ireland	Female	First year	Thin	Moderate decomposition	Dark scant intestinal contents; no upper GIT lesions. Reddened proventriculus, dark GIT contents. Lung congestion/ haemorrhage.	Neg	Pos	Pos	Pos	Undetermined	Yes – chaffinch with trichomonosis
XT0559-12	Chaffinch	Jun-12	Northumberland, England	Male	Adult	Thin	Advanced decomposition		Neg	Pos	Pos	Pos	Undetermined	Yes – chaffinch and greenfinch with trichomonosis Jun-12

Neg=negative; Pos=positive; ND=not done