HYPOTHYROIDISM: OVER-SCREENED AND OVER-DIAGNOSED

Hypothyroidism is one of the most common canine endocrine diseases, but perhaps also the most misdiagnosed[1,2] writes Dr Aine Seavers MVB MRCVS who has a particular interest in the fields of thyroid disease, toxicology and photobiomodulation

It is critically important that thyroid function tests should only be performed in dogs with physical findings and clinical signs consistent with hypothyroidism[3].

However, because of the abundance and ease of on-site testing kits, more and more dogs are being tested for this condition, not because they have signs suggestive of the condition, but simply because the serum thyroid TT4 tests are so omnipresent in so many on-site clinic blood profiles. The result of which – from what is often one test, sampled from one moment in time – is often taken at face value, and drug supplementation therapy subsequently instigated.

The problem is that the long-held view of thyroxine as a 'safe' hormone, hard to harm with, and cheap and easy to give, is under challenge. We wouldn't run dogs on an insulin, oestrogen or stilboestrol hormone trial with the quite the same laid-back nonchalance as is done with thyroid hormone; yet few are concerned about inappropriate, even life-long use of this thyroid medication in many dogs, often without a definitive diagnosis.

Granted the situation is a complicated one, not least because thyroid hormone affects metabolic processes in all body systems and so hypothyroidism potentially can produce multiple non-specific clinical signs.

A definitive diagnosis of hypothyroidism is further complicated by the lack of readily available and affordable diagnostic tests that demonstrate acceptable sensitivity and specificity.

The clinician must first recognise clinical features that are not invariably characteristic and use confirmatory laboratory tests which can be difficult to interpret, especially in the presence of non-thyroidal illness or recent drug administration. An additional complication is that analytic techniques and reference intervals for different hormones vary from one laboratory to another.

It has become quite clear that reference ranges for a wide array of clinical and laboratory measurements in canine medicine are strongly influenced by breed, and this impacts on the interpretation of values for haematology and serum biochemistry (e.g., haematocrit, leukocyte counts, cholesterol concentrations) and the results of hormone assay determinations.

For example, it has been shown that Greyhounds have higher haematocrits and higher blood viscosity than non-Greyhounds; they also have a different ability to hepatically bio-transform certain drugs, e.g., the thiobarbiturates, and have extreme echocardiographic parameters and a propensity towards hypertension. However, a suggestion to apply the reference interval (RI) of Greyhounds to other sighthounds, based on similarities, has now been found not to be applicable for many sighthounds.

With regard to total thyroid hormone or TT4, concentrations (< 17 nmol/L) are suggestive of, but not diagnostic for, hypothyroidism, as subnormal TT4 concentration can be seen in healthy, euthyroid dogs. It has been proposed that several sighthound breeds have TT4 concentrations lower than the normal canine RI. TT4 concentrations in serum are lower than in 'normal dogs' in Greyhounds, Whippets, Scottish Deerhounds, Giant Schnauzers, conditioned Alaskan sled dogs, Sloughis, Basenji and Salukis, while higher values have been reported for the Polish Owczarek Nizinnys breed. Thus TT4 concentrations by themselves have limited scope for determining thyroid function in these breeds.

The situation is thus further complicated when a specific breed society decides to screen breeding stock for hypothyroidism on the basis of an alleged inherited basis for the condition. Driven by 'abnormal' results, based on inappropriate laboratory reference intervals which do not

A Basenji at exercise. Photo courtesy of Ffire Photography.

Basenji dogs appear to require smaller doses of thyroxine for replacement therapy than 'normal dogs'.
Photo courtesy of Ffire Photography.

take breed differences into account, a situation may arise where healthy individuals are unnecessarily removed from the gene pool on the basis of such testing.
Recommendations on the use of TT4 concentrations as prognostic indicators in dogs with non-thyroidal illness further emphasises the importance of referencing breed-specific concentration intervals for each patient. The failure to address breed-specific reference intervals can see prognostics of a particular ill[4] animal so skewed that euthanasia of a survivable case occurs; such scenarios can result in legal action by owners against vets, as has already occurred in Australia.

External factors also come into play affecting serum thyroid levels

Ambient temperature can affect serum thyroid levels. Circadian rhythm and time of day can also affect levels as can age, weight and breed.
Factors such as whether the dog is an indoor or outdoor dog, as well as the time of year, if Winter or Summer, can affect the endogenous levels. A European study[5] showed "serum TT4 and fT4 concentrations had seasonal dependence in both outdoor and indoor dogs of all breeds. In dogs kept outdoors, this fluctuation was significant and quantifiably dependent on ambient temperature".
Feeding may not affect the TT4 levels, but TSH is affected by feeding in the 12 hours preceding testing in humans.
So whether you fast the patient or not, the time of day, the ambient temperature, the full medical history, the genetics of the patient, and the patient's current clinical status all must be factored into the 'how', the 'when', and 'the what now?' of thyroid blood testing.
This makes some investigators and many clinicians question whether it is indeed possible to diagnose canine hypothyroidism conclusively in some instances.
A large proportion of my general practice work in the last 20 years stemmed from somehow attracting to my practice sighthound breeder clients deeply invested in the health of the overall breed, rather than in the overall profit to be made from an individual breeding dog. Conflict arose because what the dogs were telling me as a clinician, and what the powers-that-be told me the blood tests meant, did not match up. In the end, I believed my patients, and questioned the status quo on many aspects of hypothyroid diagnosis and dosage, but especially the absence of breed-specific blood reference intervals and, more recently, the absence of breed-specific drug dose per kg guidelines and/or individualised induction regimes. The results of my investigations all have been peer-review published and cited.
As it might be helpful to other time-poor clinicians out there to have access to an abbreviated, more practice-focused version of that decade-plus of findings, I have put together a summary for other clinician colleagues.

Traditional therapeutic thyroxine supplementation trials as a diagnostic tool — maybe not!

While a therapeutic trial of thyroid supplementation is commonly used to determine the accuracy of a presumptive diagnosis of hypothyroidism, limitations exist because pharmacological actions of thyroxine can produce an improvement in 'thyroxine-responsive conditions' even in patients that are not actually hypothyroid. Such conditions can improve with thyroxine administration even when the patient is not hypothyroid.
There is a growing concern that a therapeutic thyroxine trial may not be as innocuous as was once presumed. In cases of illness and malnutrition, the body expresses 'Low T3 Syndrome'[6] in which the production of T3, the most potent thyroid hormone, is down-regulated by suppression of the enzyme that converts T4 to T3. This reduced production is considered an adaptive mechanism, whereby the body acts to limit protein loss by decreasing metabolic rate during chronic or severe illness. Thyroxine supplementation in humans with severe non-thyroidal illness is currently also controversial and the subject of prospective studies.

Adverse thyroxine supplementation reactions – more common than you think

Excess thyroxine supplementation in dogs can induce distressing side-effects including anxiety, panting, polydipsia, polyphagia, diarrhoea, pyrexia, and pruritus. Of particular concern is the patient with concurrent cardiac disease, where dose-related precipitation of heart failure has been documented. It would, therefore, seem prudent to exercise caution in supplementing dogs under similar circumstances, particularly in sighthounds which routinely function with normal lower serum TT4 concentrations than other breeds.
Thyroxine dosing issues have recently been reported in the human arena, where concerns have been raised regarding

TT4 concentrations in serum are lower in Giant Schnauzers than in 'normal dogs'.

high thyroxine doses causing increased risk of bone fracture and atrial fibrillation in elderly patients. A dose-response relationship was documented in human patients, with the requirement for decreasing thyroxine dose with age.

A significant observation is that actual hypothyroid sighthound Basenji dogs appear to require smaller doses of thyroxine for replacement therapy than 'normal dogs'. Currently recommended dose rates for thyroxine (10-20µg/kg/day) produce unacceptable clinical signs including nervousness, tremor, head-bobbing, and tachycardia when administered to Basenji, irrespective of whether they are truly hypothyroid or not. These signs occur when post-pill serum TT4 concentrations are within, or just above, the TT4 RI for normal dogs. As a result, a therapeutic trial using thyroid replacement therapy is not as straightforward and innocuous as it is in other breeds.

The current advice, when managing definitively or presumptively diagnosed cases of hypothyroidism in a Basenji or other sighthounds, is to start with a low dose (e.g., 5µg/kg once daily), and increase the dose gradually, while closely monitoring for restlessness, head-bobbing, ataxia, etc. The final tolerated dose in Basenji is expected to be approximately 25 per cent to 50 per cent of the normal recommended thyroxine replacement dose for other breeds, producing serum TT4 concentrations in the RI for Basenji, but lower than the RI for 'normal dogs'.

Genomic adversomics, a term for the genetic predisposition to adverse events, is germane to the issue of why Basenji exhibit dose-related sensitivity to thyroxine. The current drive in human medicine is to practice personalised medicine with predictive prescribing and drug doses tailored to the individual patient. This hinges on an appreciation of immunogenetics, and considerations such as knowing whether a patient is a high or low metaboliser. The Basenji breed-specific reaction to conventional recommended doses of thyroxine may represent a true genetically-programmed adversomic, potentially related to different kinetics of, or sensitivity to, thyroxine. Personal communications from other colleagues who have a significant sighthound clientele of other breeds are suggestive that this drug dose intolerance may well be the case with other sighthound breeds as well.

So, while the short half-life of T4 in normal dogs (12 to 24 hours) makes over-supplementation theoretically more difficult in dogs than in people, we now have concerns that the, as yet unknown, true pharmacokinetics of thyroxine in the Basenji or other sighthounds, compared to normal dogs, may cause significant issues with drug intolerance.

One possible way to reduce over-diagnosis is to use an rhTSH-human recombinant Thyroid Stimulation Test in dogs. This product is safe, easy and quick to use, and very accurate, but is potentially expensive if you don't know the workarounds on safely storing it long term.

The reality is that while dynamic thyroid testing (previously carried out using chemical or bovine TSH) remains the 'gold standard' for thyroid function testing[3], the test has not been routinely performed for many years due to problems

with sterility of TSH, the risk of acquiring prion disease from bovine TSH, and the general difficulty in accessing bovine TSH or chemical grade formulations.
However, these earlier TSH formulations have been replaced in recent times by rhTSH, a glycoprotein produced by genetic engineering.
This formulation appears very safe, allows for long storage (60-210 days frozen)[7,8,9], is easy to administer and, in non-sighthound breeds, the results are very easy to interpret against a wide range of diagnostic interpretative criteria.
In Australia, rhTSH kits are available on individual order from Genzyme Sydney. While expensive per initial purchase (kit costs approximately $2,000 for 2 x 1.1mg vials of rhTSH), each kit does anywhere from 9-18 dogs depending on doses used. A good scenario would be where one local practice buys the kit, then neighbouring practices purchase a couple of individual vials each to share out the initial outlay cost.
Another option is to freeze the diluted vials as soon as the product is reconstituted. Freezing the dilute product in individual small vials for up to up to thirty weeks demonstrated the same intravenous post injection levothyroxine stimulation efficacy and the same high safety margin parameters as that of freshly-constituted rhTSH, resulting in significant shelf-life extension and efficacy of this dynamic stimulation agent.

How to run a rhTSH dynamic test in your clinic

A 1.1mg vial of rhTSH (in my case, Thyrogen; Genzyme, Sydney) is freshly prepared on Day One into 1.2 mL of sterile water to produce a 0.9mg/mL solution, following the manufacturer's directions. The rest of the manufacturer's instructions are to use within 24hrs of being diluted, therefore be aware that while peer-reviewed publications show safety and efficacy beyond that date, such use would be off-label so make sure your client signed consent form is clear and up to date.
Individual aliquots (0.06mL) of this solution are drawn up into insulin syringes (100U/mL; i.e., 6U = 0.06 mL = 54 µg rhTSH). Insulin syringes are used to minimise dead space.
A blood sample is taken for pre-TT4 and pre-cTSH levels from a 12-hour fasted dog. Individual aliquots of this fresh preparation are then administered IV to the dog.
The dog returns six hours after injection and a post-rhTSH blood sample is drawn.
The syringes, red rubber capped or single dose vials, can store frozen to be subsequently defrosted in the fridge as needed, allowed to come to room temperature, and then administered I/V.
Some overseas vets and researchers added 5 or 6ml of sterile water to the original vial and then used 0.5ml of this larger dilution to decant into insulin syringes, as they found it easier to use to measure out the dose. The larger volumes have been shown to be effective when frozen up to as much as 12 weeks.
Our vials, dispensed off an initial smaller volume dilution of 1.2ml, were stored frozen, also in zero dead space insulin syringes, and capped. These individual load syringes were used effectively out to 210 days and were easy to administer straight I/V by using a zero dead space insulin syringe to gain IV access.
Several different studies use different dose regimes. Up to 16kg weight, I use 54 µg (0.06ml). I increase the dose by a

Small breeds have higher TT4 concentration than larger breeds such as the German Shepherd.

factor of two or three depending on the size of the dog. A 2009 publication[10] using a mixture of breeds recommended that dogs with concurrent disease or on medication should be tested with 150µg rhTSH (3x the dose used above) to improve differentiation between primary hypothyroidism and non-thyroidal disease. While correct, others have not found the same need to use so high a dose and a lower dose means more dogs can be tested from the same vial with better economics and no loss of accuracy.

Diagnostic interpretive criteria of rhTSH results

The rhTSH test results can be analysed using the following criteria:

a. TT4 levels throwing 1.5 x rise in TT4 levels (Dixon)
b. TT4 levels rising 24 nmol/l above baseline (Sauvé)
c. A rise in TT4 levels 20 nmol/l above baseline (Daminet)
d. TT4 levels increasing to above 40 nmol/l (Daminet)
e. TT4 levels increasing to above 45 nmol/l (Paradis).
f. Larsson's equation:

Where the discriminant function k= 0.5 x pre-TSH T4 + [post-TSH T4 – pre-TSH T4] T4 units = nm/l.
k > 30 rules out hypothyroidism,
k < 15 suggests that hypothyroidism is likely.
k values of between 15–30 are non-diagnostic or equivocal.
For sighthounds, we have found Larsson's equation[11]

applied to rhTSH testing currently shows great potential in assisting interpretation of dynamic thyroid testing results as it provides a buffer to better reflect a breed that suffers from such a normal low starting pre-stimulation TT4 value.
As a result, in the absence of clinical signs but in the presence of 'abnormal' serum hormone blood results, utilising Larsson to interpret the results of dynamic testing has allowed us take the decision to avoid initiating supplementation for the 'equivocals' at that time. Larsson allows the clinician to avoid any untoward effects of thyrotoxicosis from un-necessary drug trials or drug therapy, and aids the clinician in the decision-making process as to whether or not to supplement a particular dog.
I have a basic table of all the criteria into which I insert the results and compare against all criteria to give a quick overall view of the individual animal's test result.

Therapeutic Monitoring

In humans, monitoring serum TSH concentrations and TT4 concentrations concurrently is the preferred method of monitoring thyroid replacement therapy. A conceptually similar approach has been recommended by some veterinary researchers. The present author concurs with this approach for those breeds with a lower than 'normal' reference interval for serum TT4 concentrations as attempts to achieve a post-pill TT4 concentration achieved by normal dogs may induce clinical signs of thyrotoxicosis in these thyroxine-sensitive breeds.
Serial serum cTSH concentration determinations, with reduction of the cTSH concentration into the normal RI, provided additional evidence that thyroxine replacement therapy had been optimised in hypothyroid Basenji that only tolerated reduced oral doses of thyroxine. True hypothyroid

THYROID TESTING: COMPLICATING FACTORS TO CONSIDER

Timing of Blood Draw

- Circadian rhythm.
- Test between 10-2pm when TT4 naturally high (my preference is 10am-12pm).

Seasons

- Watch for season (and being in season).

Housing

- Whether patient housed indoors or outside.
- Ambient temperature if housed outside especially if a medium breed meant lower values in summer and higher in winter.(RCT Beagles show a different result). This differs from humans where the value is lower in cold weather.

Exercise

- Exercise level-training won't affect the values.

Work

- Police dog work, for example, has a smaller impact on hormone concentration compared to intensive sport activity.
- Endurance events are believed to affect the values. However, endurance breeds like the Alaskan Sled Dog retain lower thyroid levels even when retired, so again both the breed and lifestyle must be factored into a decision re need for therapy.

Breed

- Body size: small breeds have higher TT4 concentrations than larger breeds, e.g., French Bulldogs return higher normal TT4 values compared to a German Shepherd. Breed-specific exceptions exist, i.e., Giant Schnauzer, Deerhounds etc, so always factor breed into any interpretation.

Age

- Nursing pups have higher TT4.
- Older dog has lower TT4.

Body Condition

- Reduced in sepsis.
- Obese euthyroid dogs have higher TT4 values so an obese dog with a low indeterminate result has a higher level of suspicion of being truly hypothyroid.
- In healthy dogs >25kg, concentrations of fT4 and TT4 decrease and cTSH increases with age > 6 yrs.

Medications

- If feasible, dogs given medications known to affect thyroid function should not even have thyroid testing performed, because it is likely to result in inaccurate test interpretation and potentially unnecessary treatment" (Panciera).
- For an up-to-date review of interfering medications, download the free pdf from JIVM 2023 by Bolton & Panciera: Influence of medications on thyroid function in dogs[14].
- Drugs that don't interfere with TT4: Potassium Bromide; Pexion –at least to 18 weeks; Cephalexins.

Treatments

Photobiomodulation/Class 4 lasers can have impacts on the diseased thyroid gland. While we have been taught to avoid this area as a general rule when using laser/PBM therapy, the more recent articles now suggest that PBMT does not cause morphological changes on the healthy thyroid gland. However, in the diseased thyroid gland, PBM therapy can have a beneficial effect.
In one study in humans, use of PBMT caused a reduction in the daily dose of thyroid medication. Given that PBMT targets and modulates inflammation, and given so many thyroidal diseases are inflammatory or immune-mediated in nature, it makes sense that PBMT would be beneficial rather than detrimental in these cases.
However, if your patient is on PBM therapy for any reason be alert that the therapy can alter serum thyroid levels enough that a marginal hypothyroid case could be missed. If you do run a thyroid test, just factor in any interference in your serum blood level result (clarification: do not use the laser anywhere near any neoplastic lesion, even if it is a tumour in the thyroid gland, i.e., cats).

Basenji stabilised clinically and the initially-elevated cTSH concentrations returned to within the normal RI during appropriate replacement therapy. At personalised, optimal thyroxine replacement dosing, serum TT4 concentrations of several dogs remained lower than TT4 RI for normal dogs, but their serial serum cTSH concentration determinations show reduction of the cTSH concentration into the normal RI, providing additional supportive evidence that thyroxine replacement therapy has been optimised.

Additional Serum Blood Tests – What Not to Use

The promotion in general practice of additional expensive thyroid tests perpetuates the myth of a thyroid epidemic and is of great concern.

Concerns arise re: the validity of the promotion of the age-sensitive Thyroglobulin autoantibodies (TGAA) as a compulsory pre-breeding screen. Attention should be paid to the contemporary view of eminent endocrinologists that, although TGAA positivity is suggestive of the potential for later development of clinical hypothyroidism, it does not establish the odds of the animal having hypothyroid offspring. This is a fact that breeders need to be made much more aware of in their decision-making in regards to selecting this test in breed screening blood panels. TGAA is also an expensive test because a single determination does not establish the animal's current thyroid status and therefore is assayed annually from 12-30 months of age until the animal is five years old. TT4 and cTSH tests are also required to be run in parallel to "verify" the TGAA result. Importantly, a positive antibody test merely suggests the possibility of lymphocytic thyroiditis; it is not a thyroid function test.

The presence of thyroiditis therefore does not signify a diagnosis of hypothyroidism (nor the need to supplement in the presence of a normal TT4). Indeed, only 20 per cent of patients with a positive TGAA test will progress to a hypothyroid state within a year. In one study of 234 dogs with antithyroglobulin antibodies over a 12-month period, 20 per cent developed changes in fT4 and/or cTSH suggestive of hypothyroidism, 15 per cent reverted to antithyroglobulin antibody negative status (with no change in fT4/cTSH levels) while 65 per cent remained antithyroglobulin antibody positive (or inconclusive- with no change of fT4/cTSH).

TGAA is also not recommended in dogs older than six years yet this distinction is not observed by some laboratories. Equally, the test kit accuracy of some laboratories was affected by vaccination or oestrus in the previous 100 days, yet was often done on a bitch peri-season and/or recently vaccinated or in elderly stud males. The resultant scenario, when this test came back in vogue some years ago, was that I had distressed breeders contacting me with the need to remove important breeding lines from small gene pools on the basis of this TGAA test. I advised caution and to have their vet redo the female's test 100 days later while observing complicating factors – the bitches came back in clear. The male dogs had other testing done which came back normal. The same dogs bred well for several years after and never showed signs of hypothyroidism.

So, I don't routinely use this TGAA test and was glad to read in both 2015 (Randolf et al)[12] and 2021 (Taszkum et al)[13] that this test continues to remain less important than TT4, cTSH and FT4.

Esoteric Tests

Thyroid scintigraphy had been suggested as a more suitable 'gold standard' for determining thyroid status in dogs with low serum TT4 concentrations but is simply not practical in a general practice setting wherein the majority of thyroid testing is undertaken. Recent work suggests further studies are needed to evaluate this expensive and difficult to access modality; there are concerns that measurements made using the gamma-camera may be non-diagnostic, asymmetric uptake can occur normally, and some drug therapies interfere or suppress thyroidal uptake of technetium and alter hormone concentrations in serum.

Summary

My personal experience has made me focus more and more on TT4 paired with cTSH, and often repeated serially to see a pattern. TT4 paired with cTSH along with FBC and cholesterol as a minimum is analysed. T3 would be added for sighthound breeds.

For screening in suspected cases, I take fasting samples on a day where ambient temperature is not excessive(< 28°C) and on those days the blood is harvested before noon.

I stay with the laboratory I started with so I can minimise laboratory variables. I find out the days the laboratory runs their hormone panels – often only once a week. I then harvest my patient's screening blood test the day before, or morning of, the laboratory's assigned day to ensure my bloods are not sitting there over a week before analysis. Doing so removes a heap of variables from the result and ensures my own personal library of thyroid serum results have all been collected etc under similar conditions.

I then compare the results against the clinical status of the animal, its age, breed, size, living conditions, and previous medications and supplements.

If the results are inconclusive and I have access to rhTSH, I will do that either Day One, or some weeks later if the dog is not progressing as expected.

If the blood results and the clinical status are highly suggestive of hypothyroidism then I will instigate a clinical trial for three months.

Supplementation Therapy

For all dogs, regardless of breed, I titrate the dog up to standard doses over a two-week period (the only exception is pre-existing neurological/myxoedema coma scenarios where induction is fast). Slow titration allows you to pick up the drug intolerance or sensitive patients early in their distress.

Three months later, I post-pill test 4-6 hours later as a minimum. Sometimes, I will do a trough level as well, 12hrs or 23hrs pre-pill, depending on dose frequency.

For sighthounds, I will include post-pill TSH levels whereas for other breeds I mostly only do TT4.

If the post-pill cTSH is back to normal and the dog is clinically well but the TT4 is not in the RI for non-breed specific TT4, I don't change the dose.

I accept my approach is not standard. I simply share it with others in case it might explain hypothyroid complications and conundrums practitioners have had, for example, where the use of pre-existing protocols did not resolve the issue, as was the case for several of my patients.

Conclusion

- Think before you thyroid test any canine patient.
- Standardise how you harvest and process your samples.
- Interpret the actual TT4 tests against complicating factors as detailed in this article.

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READER QUESTIONS AND ANSWERS

1. WHEN SHOULD A TT4 SERUM BLOOD TEST BE RUN ON A CANINE PATIENT?

A. Every time a serum blood sample is obtained
B. Only when the dog shows clinical signs of hypothyroidism
C. Every time a bitch comes into estrus
D. A & C

2. GREYHOUNDS, COMPARED TO OTHER BREEDS HAVE...

A. Higher haematocrits
B. Higher blood viscosity
C. Different ability to hepatically bio-transform certain drugs e.g. the thiobarbiturates
D. Propensity towards hypertension
E. All of the above

3. WHICH OPTION IS FALSE? TT4 CONCENTRATIONS IN SERUM ARE LOWER THAN IN 'NORMAL DOGS' IN...

A. Whippets
B. Scottish Deerhounds
C. Basenji
D. Polish Owczarek Nizinnys breed

4. POTENTIAL ADVERSE DRUG REACTIONS TO A THERAPEUTIC THYROXINE TRIAL IN DOGS INCLUDE:

A. Anxiety and panting
B. Inappetence
C. Constipation
D. Tremor and head bobbing
E. A & D

5. THYROID HORMONE SERUM TEST RESULTS SHOULD BE INTERPRETED AGAINST THE ANIMAL'S...

A. Clinical status
B. Age
C. Breed
D. Living conditions
E. All of the above

ANSWERS: 1B; 2E; 3D; 4E; 5E.